Optimising grass seeding rates for improved establishment of perennial legumes in cocksfoot (*Dactylis glomerata*) swards

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Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma and, to the best of my knowledge, contains no copy or paraphrase or material published or written by any other person, except where due reference is made in the text of this thesis.

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Abstract

The effect of grass seeding rate on legume establishment was investigated in the low rainfall region of the Tasmanian midlands. Three clover species, Caucasian clover, Talish clover and red clover were paired with one of two cocksfoot species, summer active cocksfoot or winter active cocksfoot, at three different grass seeding rates 1 kg/ha, 3 kg/ha and 5 kg/ha. Successful establishment was defined as the maintenance of a clover proportion of 20-45% in the sward, and was determined using plant number. Successful establishment of all three clover species was achieved for the 5 month duration of the experiment. Clover proportions within the recommended range were recorded when Talish clover or Caucasian clover was sown with either cocksfoot species at the grass seeding rate of 3 kg/ha. The same result was achieved when red clover was paired with either cocksfoot species at the grass seeding rate of 5 kg/ha. Sowing treatments that contained red clover produced a significantly (P<0.05) greater biomass, than sowing treatments that contained Talish clover or Caucasian clover. The results of a parallel experiment conducted in a glasshouse at the University of Tasmania, highlighted several key differences between the species in morphology and development rate. A faster rate of leaf emergence, larger leaf area and larger shoot length were observed on cocksfoot seedlings than observed on Talish clover and Caucasian clover seedlings. Red clover seedling were observed to develop secondary stems, this contributed to their greater biomass production and improved performance relative to that of the other clover species. Issues of poor establishment previously reported for slow growing clover species, such as Caucasian clover and Talish clover, were overcome by pairing these species with cocksfoot at a lowered grass seeding rate.

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1 Literature Review

1.1 Introduction

Pastures play a central role in Australian agricultural systems, with greater than 50% of Australian land classified as pasture for grazing (inclusive of native, improved and irrigated pastures) (ABARES 2016). Pastures also play an important role in Tasmanian agricultural systems, with 53% of the state's agricultural land classified as improved pastures for grazing (ABS 2015). These pastures form the feed base of the wool, red meat and dairy industries, providing a relatively inexpensive feed for livestock. These industries collectively contribute over $863 million to the Tasmanian economy annually (Bennett 2017; ABS 2008). Therefore, it is important to understand how to maximise pasture production and quality, while maintaining low economic inputs. The addition and maintenance of legumes in pastures can help to achieve this goal (Peeters *et al.* 2006). Previous research has highlighted the difficulty of maintaining adequate proportion of legumes in pastures, particularly during establishment (Black *et al.* 2006b; Mills *et al.* 2015). This is in part due to differences in competitive ability between grass and legume plants for resources, such as light, water and nutrients (Walker and King 2009; Black *et al.* 2006a). Several management strategies have been identified that can modify the dynamics of a pasture during establishment, improving the growth and performance of legumes in mixed swards.

This review aims to examine: 1) the benefits legumes provide to pastoral systems in terms of biomass production and nutritional value; 2) differences between grass and legume plants in terms of their ability to compete for resources (light, water and nutrients); 3) strategies producers can apply at sowing to aid legume survival and performance during establishment. Some of these strategies include selection of compatible species, temporal or spatial separation of species, and manipulation of sowing date, grazing frequency or grass seeding rate. This review will centre on studies concerning grass seeding rate, with a focus on several key legume species.

1.2 What is a pasture?

A pasture or sward, consists of a community of individual plants that can consist of a single species (monoculture) or multiple species (mixed sward) (Langer 1990). Most pastures will include plants from the Poaceae (grass) family and/or plants from the Fabaceae (legume) family (Dear and Virgona 1996). Some grazing systems will also include forage herbs such as plantain (*Plantago lanceolate*) or chicory (*Cichorium intybus*) (Kemp *et al.* 2010). The dynamic nature of pastures results in frequent changes in species composition, these changes can become obvious shortly after sowing (Tow and Lazenby 2001). Pasture plants are selected based on their ability to remain productive under known environmental conditions (Nichols *et al.* 2012), and their level of compatibility with companion species (Annicchiarico and Proietti 2010).

1.3 Common pasture grass species in Australia

Grass plants form the sole, or largest component of most pastures. Grass plants are highly productive, well adapted to a wide range of environmental conditions, persistent and have a quick regeneration cycle (Linder *et al.* 2017). Some commonly utilised grass species in Australian pasture systems are perennial ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*) and phalaris (*Phalaris aquatica*). These species differ in their adaptation to environmental conditions and in their growth rate. Perennial ryegrass is quick to establish and highly competitive with other pasture species for resources, which makes it a very productive species under irrigation or in high rainfall areas (Phelan *et al.* 2015a). Cocksfoot and phalaris are relatively slow growing during establishment in comparison to perennial ryegrass, and display a greater drought tolerance (Clark *et al.* 2016). While grass monocultures can provide sufficient feed for livestock, the addition and maintenance of legumes within a pasture can increase its overall productivity and nutritive value.

1.4 Common and alternative perennial legume species in Australia

Despite the diversity of environmental and climatic conditions in Australia, several pasture legume species are favoured by producers. The two most commonly utilised perennial species are white clover (*Trifolium repens*) and lucerne (*Medicago sativa*). Both species are highly productive under irrigation and in the high rainfall areas of southern Australia (Nichols *et al.* 2012), however, are not well suited to all environments. Lucerne has a low tolerance to conditions of waterlogging and acidic soils (Humphries and Auricht 2001). While white clover has only a moderate drought tolerance, as after the first growing season the taproot it develops is replaced by a shallow stoloniferous root system (Annicchiarico *et al.* 2015), which makes it unsuitable in low rainfall areas in the absence of irrigation

Some suggested alternative legume species with improved drought tolerance to white clover are Caucasian clover (*Trifolium ambiguum*), Talish clover (*Trifolium tumens*) and red clover

(*Trifolium Pratense*). These species differ in their biomass production, nutritional value, rate of development and adaptation to stress. Red clover is quick to establish, and is therefore competitive against grasses for light during seedling development (Annicchiarico *et al.* 2015), it also provides a high nutritional value (Black *et al.* 2009). Talish clover has high tolerance to conditions of extended moisture stress (Hall 2013), but has a slow growth rate and low above ground biomass production during establishment (Dodd and Orr 1995). Caucasian clover is also recorded to have high tolerance to water stress (Nichols *et al.* 2012), and is slow growing during establishment (Gierus *et al.* 2012), but after this initial slow development it will produce good yields of high nutritional value (Black *et al.* 2007; Black and Lucas 2000).

1.5 Legumes and nitrogen fixation

The inclusion of leguminous plants into pastures provides several key benefits. Plants can only uptake nitrogen in the form of nitrate or ammonium (Lea and Azevedo 2006). However, much of the Earth's nitrogen is in the atmospheric form, di-nitric oxide (N_2) which is not available to plants. Legumes have the ability to fix atmospheric nitrogen into ammonia; through a symbiotic relationship formed with rhizobia bacteria housed in nodules on their roots (Phelan *et al.* 2015b). The process of nodule formation occurs in several steps. Chemical signals are traded between rhizobia present in the soil and the roots of the host plant. This involves the release of nod-factors, lipochitin oligosaccharides, by the rhizobia and the release of flavonoid signals by the roots of the host plant (Djordjevic *et al.* 2015). If in accepting these chemical signals the two are found to be compatible, the bacteria will colonise a susceptible root hair, and once inside will use the host plant's meristematic tissues to develop a nodule (Djordjevic *et al.* 2000). Energy is provided by the host plant to the bacteria which allows the fixation process to occur (Hardarson and Atkins 2003). This transformed nitrogen is then released into the soil and is available to the host plant and to other surrounding plants (Thilakarathna *et al.* 2016).

The process of nitrogen fixation by legumes can be highly effective. Clover species, white clover and red clover have both been observed to fix over 300kg/N/ha/year when planted as a monoculture (Kumar and Goh 2000; Rasmussen *et al.* 2012). Ensuring optimum growing conditions for legumes can help maximise the amount of nitrogen which is fixed (Carlsson and Huss-Danell 2003). The fixation of biological nitrogen supplied by legumes can reduce the need for the application of synthetic nitrogen fertilizers, which can provide both environmental and economic benefits (Beck *et al.* 2012). Nitrogen is a key essential nutrient included in many molecules and cellular structures, therefore a deficiency can have significant impacts on plant productivity (Kumar and Sharma 2013). The ability of legumes to fix atmospheric nitrogen is one of several factors that leads to the improved biomass production of mixed swards.

1.5.1 Biomass production

The inclusion of clover in a pasture can increase the total biomass produced. Several studies have reported greater biomass yields from pastures which include legumes, in comparison to grass monocultures (Jolliffe 1997; Gierus *et al.* 2012; Luscher *et al.* 2014; Sleugh *et al.* 2000). This increase in dry matter production could be attributed to several factors, firstly to an improvement in resource use efficiency due to the differences in root morphology between grasses and legumes (Phelan *et al.* 2015b). These differences denote a tendency for plants from these two families to draw resources from different parts of the soil profile, improving the utilisation of resources both horizontally and vertically in the soil (McLaren *et al.* 2017; Annicchiarico *et al.* 2015). This increase in biomass could also be attributed to the greater amount of plant available nitrogen which is provided by legumes (Ates *et al.* 2010), particularly in low input systems where pastures are not regularly fertilized (Luscher *et al.* 2014). The addition of biologically fixed nitrogen can also have positive effects on the nutritional value of pastures; Edwards *et al.* (1993) found that the palatability of cocksfoot was influenced by the amount of nitrogen it contains. Therefore, the addition of biologically fixed nitrogen in a pasture can improve its overall nutritional value.

1.6 Nutritional value

Grass and clover plants provide different benefits to livestock in their nutritional value. The nutritional value provided is dependent on plant part (stem or leaf), plant age and season of consumption (Malau-Aduli 2007). Energy supplied in pasture foliage is often presented as metabolisable energy (ME). Clover species, tend to provide a greater source of ME than grasses, as highlighted in a study undertaken by Fulkerson et al. (2007), who observed that red clover and white clover provided a greater ME than perennial ryegrass and prarie grass (*Bromus wildenowie*). A large proportion of pasture biomass is represented by carbohydrates, which can be separated into structural and non- structural carbohydrates. Structural carbohydrates, such as acid detergent fibre (ADF) and neutral detergent fibre (NDF), are digested more slowly and often less completely than non-structural carbohydrates (Malau-

Aduli 2007). Therefore, feed which contains elevated levels of ADF and/or NDF will lower the overall digestibility of the feed, and can decrease feed intake which can negatively affect live weight gains (Allen 2000). Clover foliage contains lower levels of ADF and NDF than grass foliage (Elgersma and Søegaard 2018). Protein is important for animal health and milk production. In general, clover foliage also provides greater crude protein levels than grasses. Black *et al.* (2007) reported Caucasian clover and white clover to have crude protein levels of 304 g/kg/$\mathrm{DM}^{-1}$and 286 g/kg/DM^{-1}, significantly greater than that of perennial ryegrass at 234 g/kg/DM^{-1}. While, Sturludóttir *et al.* (2014) reported that mixed swards that include legumes provide greater crude protein levels than observed in grass monocultures

There are also differences observed between different grass species and different clover species in their nutritive value. Turner *et al.* (2006) observed cocksfoot to present higher crude protein levels and lower ADF levels than perennial ryegrass. While a study by Mouriño *et al.* (2003) noted that mixed swards which contained Caucasian clover displayed a greater nutritive value presenting a greater crude protein level, digestibility and lower NDF than mixed swards which contained red clover. Pasture species also differ in their macro and micro nutrient contribution, clover species tend to contain greater levels of calcium, magnesium, copper and zinc than grass species, while grasses contain greater levels of iron (Blackwood 2007).

Clover tends to be more palatable to livestock than grass. Livestock display a greater voluntary intake of clover from mixed swards (Michell 1972), and display a preference for swards containing higher proportions of clover (Phelan *et al.* 2015b). However, the excess consumption of clover foliage, in particular red clover or Caucasian clover can lead to bloating (Annicchiarico *et al.* 2015; Phelan *et al.* 2015b; Mouriño *et al.* 2003) that can negatively affect animal performance, so producers need to monitor livestock intake.

The nutritional benefits provided by clover are enhanced by the proportion of clover within the sward. The recommended proportion of legumes in a sward is 20-45% to provide a sufficient contribution of plant available nitrogen and nutritional benefit (Thomas 1992).

1.7 Competition and interaction between pasture grasses and legumes

Competition for resources between pasture plants begins early in seedling development. Competition is defined by Begon *et al.* (1996) as “the interaction between individuals, brought about by a shared requirement for a resource in limited supply, and leading to a reduction in the survivorship, growth and/ or reproduction of at least some of the individuals concerned.”. The intensity of competition is affected by the initial availability of resources, and the planting density at establishment (Langer 1990). Seedlings will experience competition earlier in their development when plant density is high, as there is a greater number of individuals competing for the same resources (light, water, nutrients etc.). Tow and Lazenby (2001) reported that a high plant density resulted in the development of smaller seedlings that had a poorer competitive ability. Further to this, Lee *et al.* (2017) observed that as seedlings developed and increased in size under conditions of high plant density, there was a natural reduction in seedling number as the availability of resources placed limits on population size.

Grass species in general have been recorded to be strong competitors during establishment, and highly persistent (Mills *et al.* 2015). A review by Linder *et al.* (2017) argues that grasses tend to be well adapted to a wide range of environmental conditions, have the ability to respond well to stress events and have a quick regeneration cycle. This provides them with an ability to compete strongly for resources and persist. It is suggested that in general clover species have a narrower range of tolerated environmental conditions, which leads them to be outcompeted by grasses when planted in optimum growing conditions (Phelan *et al.* 2015b).

Several studies have explored the impacts of competition for resources applied to plants above ground (light) and below ground (nutrients and water). Competition applied in either of these zones appeared to affect the productivity of plants differently, depending on the age/size of the plant, and the strength of the competition pressure applied. Martin and Field (1984) noted that plant development stage affected the severity of the impacts of competition above or below ground. They observed that initially, competition placed on seedlings in the root zone had a greater effect on plant productivity than when competition applied to seedlings in the shoot zone. However, as seedlings aged this trend was reversed. This could be assumed to be due to the increase in plant size as seedlings develop, leading to greater potential for shading and an increase in competition for light (Kiaer *et al.* 2013). Tow and Lazenby (2001) noted that as the level of competition for resources below ground decreased competition for light increased. Therefore, it could be argued that when adequate water and nutrients are available; a plant’s ability to compete for light will be what dictates plant performance.

Competition applied in the root zone can also negatively affect plant performance. Walker and King (2009) reported competition applied in the root zone had a significant negative affect on plant performance in terms of shoot biomass. They observed that when clover plants were placed under conditions of resource competition from grass plants in the root zone clover biomass was significantly reduced when compared to those grown in a monoculture. In contrast, no significant difference was recorded between clover seedlings grown in a monoculture and those placed under conditions of competition in the shoot zone. This is further highlighted by the review of Kiaer *et al.* (2013), who observed that when plants were grown on increasingly nutrient poor soils, competition applied in the root zone had a compounding negative effect on biomass scores. Therefore, by ensuring adequate availability of nutrients and water during early seedling growth, with the addition of fertilizers and irrigation, producers can minimise competition. Kiaer *et al.* (2013) also noted that competition tended to have a larger effect if plant size was small, or if the competitor was a grass species, highlighting the disadvantage slow-growing legume seedlings have in mixed swards during establishment. Therefore, management techniques should be applied to minimise and delay conditions of competition, which can be achieved by lowering the grass seeding rate to reduce the density of grass seedlings during early development (Black *et al.* 2006b). Competition dynamics between grass and legume plants are complex and change over time as plants develop.

1.7.1 Plant development stage

Plant development stage and the speed of development influences a plant's ability to compete for resources such as light, nutrients and water. This is best seen during establishment, as rapid germination and high seedling vigour can provide an initial advantage in accessing resources (Langer 1990). Both seedling vigour and germination rate have been reported to be positively correlated with seed weight (Black 1958). Seeds with a higher seed weight have a larger energy reserve, which when effectively utilised leads to quick germination and establishment (Cullen 1964). Moot et al. (2000) reported that pasture species Italian ryegrass (*Lolium multiflorum*) and red clover presented higher mean seed weights at 5.14 mg and 3.59 mg, than cocksfoot at 0.68 mg. These species displayed a more rapid germination and higher initial seedling weights than cocksfoot. This provides a competitive advantage for light and other resources for these species during early development. In contrast, the growth of Caucasian clover and Talish clover seedlings is recorded to be slow in comparison to other legume species and companion grasses (Black *et al.* 2006a; Walker and King 2010; Dodd and Orr 1995). In response to these observations, recent breeding programs for these species have centred on traits such as seedling vigour (Nichols *et al.* 2012).

Though plants may have a poor competitive ability at establishment, this may change as the plant develops. This is highlighted in the relationship of perennial ryegrass and Caucasian clover in a mixed sward. Black *et al.* (2006b) observed rapid establishment and growth of perennial ryegrass seedlings which led to the suppression of Caucasian clover seedlings. At the conclusion of the 12 month experiment Caucasian clover represented only 8% of the total biomass in the sward. However, it appears that the competitive ability of Caucasian clover improves three years after sowing, when Caucasian clover was observed to be strongly competitive against perennial ryegrass in irrigated and dryland conditions (Moss *et al.* 1996; Seguin 2007; Black and Lucas 2000). One possible explanation for the improvement in the competitive ability of Caucasian clover is its ability to develop an extensive system of rhizomes (Kim *et al.* 2017). The development of efficient root systems can affect a plants ability to access and compete for resources in the soil.

1.7.2 <u>Below ground competition:</u>

1.7.2.1 <u>Nutrients</u>

The botanical composition of a pasture is affected by the availability of nutrients, and ability of plants to compete for them. Grasses are known to be strongly competitive for nutrients such as nitrogen, phosphorus and sulphur due to the high surface area and wide exploration of their fibrous root systems (Evans 1977). Perennial ryegrass in particular is highly competitive for soil nitrogen, and able to extract large proportions of nitrogen when it is added to the soil as fertilizer (Rasmussen *et al.* 2012). This can lead to rapid growth of grass plants, as highlighted by Enriquez-Hidalgo *et al.* (2016) and Smith *et al.* (2015), who noted a significant increase in the contribution of perennial ryegrass to the total biomass production in a mixed sward under treatments of high nitrogen. In turn, they observed a decrease in the proportion of legume biomass. This could be attributed to several factors; one being a decline in nitrogen fixation, as high rates of applied inorganic nitrogen have been noted to decrease nitrogen fixation (Ledgard and Steele 1992) or due to a decline in the availability of light as grass plants extend into the canopy (Stern and Donald 1962b). A review by Carlsson and Huss-Danell (2003) observed that around 80% of fixed nitrogen is utilised by clover plants when sown in mixed swards. While, Hartwig *et al.* (1994) reported that the proportion of biologically fixed nitrogen utilised by clover plants increased, when placed under conditions of competition for soil nitrogen.

The ability of a plant to compete for, and gather phosphorus from the soil can affect the botanical composition of a pasture. Root morphology can dictate the level of efficiency of phosphorus uptake from the soil, as determined by specific root and root hair length (Yang *et al.* 2017). These parameters can determine how effective roots are in acquiring phosphorus from soil profile. Grasses, bearing a fibrous root system, have improved phosphorus efficiency to clovers (Evans 1977). This improved efficiency leads to a lower requirement of critical phosphorus by grasses than clovers to achieve maximum plant growth (Haling *et al.* 2016). Both of these traits should provide a natural competitive advantage to grass plants under environments with limited phosphorus availability.

However, there are several management techniques producers can apply to decrease the difference in competitive abilities for phosphorus uptake. The study of McLaren *et al.* (2017) highlighted that grass plants tend to extract phosphorus throughout the whole soil profile, while clover plants will extract phosphorus located near to the soil surface. Therefore, applying phosphorus as a top dressing helps to maximise the potential phosphorus gain by clovers improving their ability to compete for it. The maintenance of adequate phosphorus levels during seedling growth can also aid the ability of clover plants to remain competitive during establishment. Bailey *et al.* (1999) investigated the effects of several available nutrients on clover seedling growth and observed that phosphorus availability had the greatest impact on seedling performance. Therefore, ensuring that adequate levels of phosphorus are applied to the soil at sowing can aid legume performance and competitive ability during establishment. This study also observed the positive impacts that liming can have on the increased availability of phosphorus and other essential plant nutrients.

Grass plants have also been recorded to have a greater ability to extract sulphur from the soil than clover plants. Walker and Adams (1958) observed that grass plants extracted 98% of available sulphur in sulphur poor soils. Sulphur molecules are required by legumes for nitrogen fixation, so a deficiency negatively effects the amount of nitrogen fixed and their biomass production (Varin *et al.* 2010).

Low soil pH and the low availability of key nutrients such as molybdenum can negatively affect nitrogen fixation, legume performance and competitive ability in mixed swards. Low soil pH can inhibits nodulation, as many strains of rhizobia are intolerant to conditions of acidity (Mulder and Veen 1960). The number of functioning nodules present on clover plants has been observed to decline as pH decreases (Hackney *et al.* 2017). The process of nodulation is hindered in acidic soils due to the high cation balance which can lead to confusion in the initial signalling between the rhizobia and host plant (Ferguson *et al.* 2013). Low nodule number and decline in nitrogen fixation can reduce the ability of clovers to compete with grasses in a mixed sward. Low soil pH also negatively effects the availability of molybdenum in the soil. This is an issue for leguminous plants, as molybdenum forms part of nitrate reductase enzyme which is used to transform atmospheric nitrogen into ammonia (Bailey *et al.* 1999). By increasing the level of molybdenum in acidic soil, Adhikari and Missaoui (2017) observed that the number of nodules could be increased allowing an increased potential for nitrogen fixation and thus an improved competitive ability of legumes.

1.7.2.2 Water

Differences between grasses and legumes in their ability to access adequate water in environments with limited supply leads to competition. Root morphology dictates the ability of plants to access water from different parts of the soil profile (Annicchiarico *et al.* 2015) and therefore, their ability to compete for available water when in limited supply. Guobin and Kemp (1992) observed that the comparatively shallow root system of white clover contributed to its lesser competitive ability to obtain water than against phalaris when grown under conditions of water stress. Similarly, Caucasian clover was shown to be more persistent than white clover in conditions of low moisture availability due to the extensive taproot it develops (Black and Lucas 2000). Clover plants can have a competitive disadvantage under conditions of moisture stress as their ability to fix nitrogen is negatively affected (Langer 1990). The movement of nitrogen through the soil is also negatively affected by low moisture levels (Thilakarathna *et al.* 2016). This provides a competitive advantage to grasses that bear a more extensive root system. Thomas (1984) observed that although moisture stress negatively affected the foliage growth of both clovers and grasses, clovers were more strongly affected. This decreased their ability to compete for light during times of low water availability, and was further magnified when water was available again.

1.7.3 Above ground competition

1.7.3.1 Light

The capture of light and its transformation into carbohydrates during the process of photosynthesis is vital for plant growth and development (Paul and Foyer 2001). Therefore plants need to be able to compete effectively for light. There are several factors which dictate the amount and quality of light which is intercepted; leaf number, leaf size and leaf height within the canopy. Leaf height is mostly determined by a plant’s morphology, whether upright or prostrate, but can change when exposed to competition, as highlighted by Walker and King (2009) who observed increases in petiole length of Caucasian clover when in competition with perennial ryegrass.

Grass plants have an upright morphology, meaning the height of leaves in the canopy will increase as the plant develops (Stern and Donald 1962a). This gives grasses a competitive advantage for light as they experience a reduction in shading (Stern and Donald 1962b). Clover species differ in their morphology, some clover species such as red clover tend to be upright in growth habit developing branches that can reach the top of the canopy (Annicchiarico *et al.* 2015). This allows this species to compete well against companion grasses for light. However, an upright morphology can increase vulnerability to grazing as meristematic regions are elevated to a height where they can be consumed by livestock. Plant breeders have responded to this issue by developing prostrate red clover cultivars such as Rubitas that are less vulnerable to damage during grazing (Nichols *et al.* 2012).

Both leaf number and leaf size influence leaf area, and clover species differ in their investment into either trait to maximise leaf area. Several studies have highlighted the greater size of Caucasian clover and red clover leaves, comparable to white clover. Black *et al.* (2009) observed that the maximum leaf size for red clover was three times that of white clover. While Black *et al.* (2006a) noted no significant difference in leaf area between white clover and Caucasian clover plants despite white clover producing three times the number of leaves produced by Caucasian clover. Leaf number influences leaf area and increases over time, therefore, the rate of leaf emergence is important to maintain a plants competitive ability for light.

1.7.3.2 Rate of leaf emergence and leaf area

The difference in leaf emergence rate between grasses and legumes can affect the ability of plants to compete for light. The rate of leaf emergence is correlated with temperature, as reported by Arnott and Ryle (1982), they observed an increase of 5 °C can increase the rates of both leaf expansion and leaf emergence in red clover and white clover. This is due to the positive relationship between temperature and enzyme function which in turn influence plant growth rate (Arnold and Monteith 1974). Several studies have investigated differences in plant development between pasture species. Most studies use the concept of degree days (°Cd) or thermal time to quantify differences in development. This concept uses an understanding that each plant species has several growth thresholds in relation to temperature, firstly a base temperature below which no growth will occur, for temperate pasture species this is between 0-5 °C, and an optimum temperature at which maximum growth can occur when no other factors are limiting, for temperate pasture species this is 25 °C (Moot *et al.* 2000; Black *et al.* 2006a).

The number of degree days required to reach different development stages for Caucasian clover, white clover and perennial ryegrass was investigated by Black *et al.* (2006a). It was observed that there was little difference in the number of degree days required for germination and the production of primary leaves. However, Caucasian clover required nearly double the number of degree days in comparison to the other two species to produce secondary leaves. This suggests that Caucasian clover will have a low competitive ability for light interception during early seedling development, presenting a lower leaf area than its companion species. Results of a similar study by Walker and King (2009) observed a higher leaf development rate for Caucasian clover, they argued that this was due to the regular application of fertilizer to plants over the course of their experiment, highlighting the potential to increase the rate of leaf emergence through optimising plant nutrition. Black and O'Kiely (2007) investigated differences in phyllochron (number of degree days occurring between sequential emergence of leaves on the main stem (Bonhomme 2000)) between several pasture species. They reported that the phyllochron for red clover and Caucasian clover was significantly greater than that of perennial ryegrass and white clover. This further indicates the slow growth rates of these legume species, and the potential disadvantages they may experience during establishment. The allocation of carbohydrates to leaves also influences the rate of leaf emergence.

1.7.3.3 Carbohydrate partitioning

Carbohydrate partitioning dictates the proportion of total shoot biomass and, by extension, leaf area. Several studies have observed differences in carbohydrate partitioning to affect plant performance in a pasture. Laberge *et al.* (2005) observed a significant difference in carbohydrate partitioning between white clover and Caucasian clover, with the latter investing greater biomass into fewer leaves with a greater weight and leaf area during the seedling stage. This lead to comparable shoot biomasses between the two species though white clover presented a greater number of leaves. A difference is carbohydrate partitioning can be better highlighted by differences in root to shoot ratio. Black *et al.* (2006a) argue that a preferential allocation of carbohydrates to plant roots rather than leaves and shoots which leads to the low competitive ability of Caucasian clover seedlings. The work of Walker and King (2009) further highlighted the tendency of Caucasian clover to partition a significant amount of biomass into its root system during the first two years of growth, maintaining a high root to shoot ratio. However, they argued that it was not only a difference in shoot biomass which lead to the clovers' poor competitive ability, rather they argue that it is a combination of poor resource allocation and increased competition for resources in the root zone between companion plants.

1.8 Management practices to improve legume establishment

There are several practices which can be applied at sowing to aid the competitive ability of legumes during establishment. Producers can select pasture species with compatible growth habits, use temporal or spatial separation of species or can manipulate sowing date, grazing regime or seeding rates.

1.8.1 Species diversity

Adequate clover establishment can be achieved by pairing multiple clover species with a single grass species. Hurst *et al.* (2000) highlighted the success of this technique achieving successful clover establishment, at 55% clover representation in the sward, by pairing red clover, white clover and Caucasian clover with either perennial ryegrass or timothy (*Phleum pratense*). Riday and Albrecht (2012) were also successful in utilising this technique to achieve successful clover establishment and adequate representation of clover in the sward over multiple seasons. They paired Caucasian clover and red clover with three different grass species cocksfoot, phalaris and tall fescue (*Festuca arundinacea*), recording clover proportions in the sward of greater than 50%. This technique was successful over several seasons due to the difference in growth rate between the two clover species. Caucasian clover is known to have slow initial growth for the first two seasons reaching full productivity in the third season (Moss *et al.* 1996). In contrast red clover which is quick growing during early growth but only remains productive for three years (Annicchiarico *et al.* 2015). In sowing a mix of legume species the potential of legumes to fill multiple niches in the pasture system and better exploit resources is improved, this facilitates a greater potential for nitrogen fixation and plant performance, benefiting all plants in the sward (Brophy *et al.* 2017).

1.8.2 Species compatibility

Successful species pairing greatly influences the persistence of species in the sward both during establishment, and for the long term. Black and Lucas (2000) observed Caucasian clover to be more compatible with cocksfoot than white clover when grown under conditions of extended water stress, as both Caucasian clover and cocksfoot are known to be highly drought tolerant (Nichols *et al.* 2012; Culvenor *et al.* 2016) and to draw water from different parts of the soil profile due to their contrasting root systems (Evans 1977), which makes this species highly compatible under conditions of low rainfall. Similar characteristics characterise the suitability of Talish clover with winter active cocksfoot under conditions of low rainfall (Hall 2013).

1.8.3 Grazing

The application of grazing during pasture establishment can limit the height of grass leaves in the canopy, therefore, aiding the interception of light by slower growing legume plants lower in the canopy (Tow and Lazenby 2001). Managed grazing of young pastures can ensure uniformity of leaf height, minimising any natural difference in leaf height between grass and legume seedlings. Similarly, rotational grazing can be applied when legumes are over sown into a pasture, both before sowing and during early seedling growth to aid legume establishment (Tracy *et al.* 2015). Manipulation of sowing dates of pasture species can achieve similar outcomes.

1.8.4 Sowing date

The time of year that a pasture is sown can provide an advantage to the growth of some species while disadvantaging others. Caucasian clover has been observed to require a greater number of °Cd for seedling development (Moot *et al.* 2000), therefore a spring sowing rather than an autumn sowing can maximise clover seedling development and competitive ability during establishment (Black *et al.* 2006b). This is a cheap and effective way to aid legume seedlings during establishment.

1.8.5 Temporal separation of species

Delayed sowing of grass species can be used to improve the competitive ability and performance of clover seedlings during establishment. This technique was observed to be effective in studies undertaken by Walker and King (2010) and Hurst *et al.* (2000). Both observed clover plants to represent greater than 50% of the total biomass at the conclusion of the first growing season, when grass seed was over sown in the pasture 6 months after clover seedlings. However, Walker and King (2010) observed that if sowing was delayed past the three leaf stage on clover seedlings that grass seedling was negatively affected due to the high competitive ability of the established clover seedlings. Yet the practice of delayed sowing may not be readily adopted by producers, as there would be the added expense for a second sowing, and there may be damage to the pasture and soil structure resulting from the movement of machinery over soils and already established plants.

1.8.6 Spatial separation of species

Spatial separation of species by drilling seeds into alternate rows or blocks has been observed to decrease competition between companion species during establishment. Sharp *et al.* (2013) compared legume biomass during pasture establishment when planted in a mixed sward or when species were separated into neighbouring blocks. It was observed that when legumes were separated from grasses legume biomass and seedling number was greater, this may have been due the reduced competition for resources such as light in the sward (Stern and Donald 1962b). However, this sowing arrangement may limit the transfer of biologically fixed nitrogen from legumes to grasses, decreasing the benefits of legumes to the pasture in terms of increased productivity. Hurst *et al.* (2000) separated perennial ryegrass and clover species into alternate drill rows, however, despite the separation the clover species still performed poorly providing only 21 - 15 % (inclusive of red clover, Caucasian clover and white clover) of the total biomass. This indicates that a single row space is not sufficient to mitigate the strong competitive ability of perennial ryegrass, it is suggested that this technique could be applied in combination with a reduced grass seeding rate.

1.8.7 Seeding rates

The grass seeding rate can be reduced to decrease the competition pressure between legume and grass seedlings in mixed swards. Lowered grass seeding rates decrease the initial density of grass plants allowing the development of legume plants before interspecific competition for resources begins. Perennial ryegrass is reported to be highly competitive during establishment (Cullen 1964), and several studies have been undertaken to observe the effects of grass seeding rate on the level of competition applied to companion legumes. Hurst *et al.* (2000) suggested that perennial ryegrass should be sown at rates of less than 8kg/ha to allow the establishment of red clover and white clover. However, they observed that a low grass seeding rate (3.5 kg/ha) was not sufficient to facilitate the establishment of Caucasian clover with perennial ryegrass with Caucasian clover contributing less than 1% of the total biomass 16 months after sowing. Black *et al.* (2006b) observed similar results when sowing Caucasian clover with perennial ryegrass at the slightly lower grass seeding rate of 3kg/ha, noting Caucasian clover to represent 8% of the total biomass 14 months after sowing. In the same experiment successful establishment of white clover was achieved when paired with perennial ryegrass at grass seeding rates of 3-8 kg/ha. The results of both of these studies indicate that a lowered grass seeding rate can be effective at aiding legume establishment, but is not alone sufficient, the compatibility of the species must also be considered. However, utilising this technique may negatively affect the total biomass production, as there will be fewer plants present in the sward.

1.9 Application

The difficulty of establishing slow growing legumes such as Caucasian clover into mixed swards has been well documented (Black *et al.* 2006b; Hurst *et al.* 2000; Mills *et al.* 2015). Several studies have been undertaken to try and observe differences in plant morphology and development that may convey the low competitive ability of these species during establishment (Walker and King 2009; Black *et al.* 2006a; Black and O'Kiely 2007). The majority of studies have centred on Caucasian clover, with no similar studies recorded for Talish clover. Talish clover has been reported to be highly persistent and drought tolerant, attributes shared by Caucasian clover (Hall 2013; Nichols *et al.* 2012). These attributes make these species well suited to the low rainfall regions of southern Australia where commonly utilised legume species such as white clover fail to persist (Black and Lucas 2000). The high drought tolerance of cocksfoot (Culvenor *et al.* 2016) and it's slow growth rate, in comparison to other grass species such as perennial ryegrass (Moot *et al.* 2000), make it a suitable companion grass for Caucasian clover and Talish clover under low rainfall conditions.

Previous studies have achieved improved establishment of legume species utilising a lowered grass seeding and a compatible grass species (Hurst *et al.* 2000; Black *et al.* 2006b). It is suggested that the issue of poor establishment previously observed in pastures containing slow growing clover species such as Caucasian clover or Talish clover, may be overcome by pairing these species with a compatible grass species such as cocksfoot at a lowered grass seeding rate.

2 Optimising grass seeding rates for improved establishment of perennial legumes in cocksfoot (*Dactylis glomerata*) swards

3 Introduction

Improved pastures for grazing represent 53% of Tasmania's agricultural land (ABS 2015). These pastures form the feed base of several key industries, including the dairy, wool, and red meat industries. The Tasmanian midlands region contains a significant area of this pastoral land. Species selected for these pastures need to be tolerant to extended periods of moisture stress, as much of the region is classified as a low rainfall area (DPIPWE 2012). Cocksfoot is a grass species well suited to these climatic conditions, as it has a high drought tolerance (Culvenor *et al.* 2016) and is already widely used in Tasmania. White clover, which is one of the most commonly sown legume species in Tasmania, is not suited to these conditions due to its poor drought tolerance. Therefore, alternative legume species with superior drought are required for this region. Caucasian clover and Talish clover are suitable alternative species, as both develop a substantial taproot which conveys high drought tolerance compared to the shallow stoloniferous root system developed by white clover after the first growing season (Nichols *et al.* 2012; Annicchiarico *et al.* 2015).

The inclusion of legume species into pastures provides several key benefits. The ability of leguminous plants to fix atmospheric nitrogen increases the productivity of a pasture, while the high nutritional value of their foliage increases the value of the feed produced (Peoples and Baldock 2001).The recommended proportion of legumes in a pasture is 20-45% to maximise these benefits (Thomas 1992). However, maintaining this proportion of legumes can be difficult, particularly during pasture establishment. Caucasian clover and Talish clover have both been observed to be slow growing during early seedling development (Black and O'Kiely 2007; Dodd and Orr 1995). This slow development rate has been suggested as a reason for the poor establishment of Caucasian clover in mixed swards (Mills *et al.* 2015; Black *et al.* 2006b).

Several studies have been undertaken to better understand how differences in plant morphology and development rate influence the competitive ability of grass and clover seedlings in mixed swards. These studies have compared the potential for light interception, carbohydrate allocation, and the rate of secondary stem development between grass and clover species (Black *et al.* 2006a; Walker and King 2009; Black and O'Kiely 2007). These characteristics provide potential explanations for the poor establishment of Caucasian clover. The majority of grass species are more competitive for resources such as light, water, and nutrients compared to clover species (Phelan *et al.* 2015b). Grass plants present an upright growth habit that allows them to intercept a greater proportion of available light in the sward (Stern and Donald 1962b). Grass plants also utilise a fibrous root system that is more effective at extracting nutrients and water from the soil, compared to the taproot system utilised by many clover species (Evans 1977).

These differences are magnified as the plant density increases (Langer 1990). A high plant density results in smaller seedlings and the loss of the weakest seedlings from the sward (Lee *et al.* 2017; Hill and Mulcahy 1995). Plant density during pasture establishment is related to the initial seeding rates. Therefore, decreasing the grass seeding rate will lower the density of grass seedlings, and potentially improve the performance of legumes. This technique has been successful in facilitating the establishment of red clover and white clover in perennial ryegrass swards, as these species present a similar growth rate (Black *et al.* 2009). When the same technique was applied to Caucasian clover in perennial ryegrass swards, clover establishment was unsuccessful (Hurst *et al.* 2000; Black *et al.* 2006b). Perennial ryegrass has a more rapid development rate than Caucasian clover, which may have resulted in the poor establishment (Black *et al.* 2006a). The results of these studies indicate that grass and clover species must present a comparable growth rate for this technique to be successful.

Cocksfoot is suggested as a suitable companion grass for both Caucasian clover and Talish clover, because it is highly drought tolerant and slow growing during establishment (Moot *et al.* 2000).

It was therefore hypothesised:

1) That successful legume establishment (maintenance of a legume proportion of between 20-45%) would be achieved when Talish clover or Caucasian clover are paired with cocksfoot at a lowered grass seeding rate.

2) That both the grass/ legume pairing and the grass seeding rate will affect the plant biomass produced.

3) That there would be quantifiable differences in plant morphology (plant height, leaf area and biomass) and development rate between the grass/ legume species, which will affect their ability to intercept and compete for light.

4 Methods

4.1 Field experiment

4.1.1 Climate and soil

An experiment was conducted in the Tasmanian midlands at the University of Tasmania farm (53°46’E, 526°10’N) beginning in October 2017 and concluding in February 2018. Temperature and rainfall data was taken from the closest weather station, located at the Hobart aerodrome 4 km from the site. During this period, the mean maximum and mean minimum temperatures fluctuated from 9.6 °C to 24.7 °C, slightly higher than the long-term average temperatures which ranged from 7.8 °C to 22.6 °C (**Table 1**). A lower monthly rainfall was recorded during October, November, and January in 2017-18 in comparison to the long-term average monthly rainfall (**Table 1**). Supplementary irrigation was applied for the duration of the experiment to ensure successful germination and seedling establishment. Irrigation was applied twice weekly for the first four weeks after sowing, and then applied as needed as dependent on soil moisture (Appendix 1).

Table 1. Mean maximum and mean minimum temperature (°C) and total monthly rainfall (mm) for 2017-18 and long-term average at closest weather station.

	Mean maximum temperature (°C)		Mean maximum temperature (°C)		Total rainfall (mm)	
Month	2017-18	Long-term average	2017-18	Long-term average	2017-18	Long-term average
October	20.4	17.4	9.6	7.8	18.6	43.2
November	22.9	19.2	11.3	9.5	32	39.1
December	22.9	20.8	13.2	10.9	98.2	51
January	24.7	22.6	13.8	12.2	25.6	41.1
February	22.2	22.5	12.3	12.2	42.6	32.5

Data: (BOM 2018a; BOM 2018b)

Prior to sowing, a systematic zigzag transect sampling technique was applied to gather thirty soil cores from the site, and then aggregated into one sample. The cores were taken to a

10 cm depth at 10 m intervals. A sub sample was sent for nutrient analysis, and from these results fertilizers were added at the rate of 150 kg/ha of N-P-K-S 8-4-10-11 to ensure nutrients were not limiting.

Table 2. Observed and recommended soil nutrient levels and soil pH.

Nutrient	pH Level (H_2O)	Ammonium Nitrogen (mg/kg)	Nitrate Nitrogen (mg/kg)	Phosphorus Colwell (mg/kg)	Potassium Colwell (mg/kg)	Sulphur (mg/kg)
Level recorded at the site	6.9	3	12	120	310	9.6
Recommended level	6.0-7.5	2-10	10-50	200-500	245-400	12-45

(Baker and Eldershaw 1993; APAL Agricultural Laboratory 2014)

4.1.2 Seed treatment

Clover seeds were scarified and inoculated prior to sowing as per breeder recommendations (Hall and Hurst 2012d; Hall and Hurst 2012e; Hall and Hurst 2012a). Group B rhizobia were used to inoculate red clover and Talish clover seeds, while Caucasian clover specific rhizobia, strain cc283b was used to inoculate the Caucasian clover seeds. Clover seeds were pre-inoculated using slurry inoculation and lime pelleting (Drew *et al.* 2012).

4.1.3 Experimental design

A randomised complete block design was utilised with four blocks. This design was selected to minimise any potential effects of slope at the site. Each block contained 28 plots of a 5 m x 1.5 m size. There were 28 sowing treatments which comprised of: red clover sown at 5 kg/ha paired with summer active cocksfoot at three seeding rates 1 kg/ha, 3 kg/ha and 5 kg/ha; red clover sown at 5 kg/ha paired with winter active cocksfoot at three seeding rates of 1 kg/ha, 3 kg/ha and 5 kg/ha; Talish clover sown at 5 kg/ha paired with summer active cocksfoot at three seeding rates of 1 kg/ha, 3 kg/ha and 5 kg/ha; Talish clover sown at 5 kg/ha paired with winter active cocksfoot at three seeding rates of 1 kg/ha, 3 kg/ha and 5 kg/ha; Caucasian clover sown at 8 kg/ha paired with summer active cocksfoot at three seeding rates of 1 kg/ha, 3 kg/ha and 5 kg/ha; Caucasian clover sown at 8 kg/ha paired with winter active cocksfoot at three seeding rates of 1 kg/ha, 3 kg/ha and 5 kg/ha. Each species was also sown as a monoculture to act as a control: Caucasian clover at 8 kg/ha; Talish clover at 5 kg/ha; red clover at 5 kg/ha; winter active cocksfoot sown at 1 kg/ha, 3 kg/ha and 5 kg/ha; and summer active cocksfoot at 1 kg/ha, 3 kg/ha and 5 kg/ha. One plot remained empty to fill the experimental design. The seeding rates for Talish clover cv. Permatas and red clover cv. Rubitas were as per the breeders recommendations (Hall and Hurst 2012e; Hall and Hurst 2012d), while the seeding rate for Caucasian clover cv. Kuratas was selected based on the recommendations of (Black *et al.* 2006b) who recommend an elevated seeding rate to improve establishment. The three seeding rates selected for the two grass species, summer active cocksfoot cv. Megatas and winter active cocksfoot cv. Uplands, all fell within the breeders recommended seeding rates (Hall and Hurst 2012c; Hall and Hurst 2012b). Seeds were direct drilled into a fine seed bed using an Ojyard precision direct drill, into rows at a 15 cm spacing (**Figure 1**).

Figure 1. Drilling seeds at the experimental site.

4.1.4 <u>Weed and insect control</u>

Several control measures were applied to limit the effects of weed and insect interference on seedling growth. The site was sprayed with herbicides twice before sowing (**Table 3**). However, a significant wild radish (*Raphanus raphanistrum*) population became established shortly after the seedlings were sown. Due to the diversity of the sown species herbicides could not be applied to control these weeds. Therefore, the site was mowed to a height of 10 cm 58 days after sowing to allow light to reach the pasture seedlings. Insecticides were also applied to control red legged earth mite and slug populations 2 weeks after sowing (**Table 3**).

Table 3. Date of application, rate and brand of herbicide and insecticide.

Date	Herbicide	Rate
31/07/2017	Roundup Ultra Max	1.25L/ha
31/07/2017	L.V.E. Agrotone	700 mL/ha
31/07/2017	Dicamba	200 mL/ha
03/10/2017	Roundup PowerMax 540g/L	3L /ha
03/10/2017	Activator surfactant	100ml/ 100L
Date	**Insecticide**	**Rate**
24/10/2017	Talstar	50ml / ha
24/10/2017	Metarex	5kg/ha

4.1.5 Measurements

Leaf number

One plant of each species was randomly selected from each plot and marked. Leaf number was recorded for these plants weekly, beginning five weeks after sowing, and continuing for 11 weeks. Only fully expanded leaves were counted.

Plant population dynamics

A 50 cm x 50 cm (0.25m^2) area was randomly selected within each plot and marked. The number of sown species in this area was recorded fortnightly, commencing eight weeks after sowing and continuing for 12 weeks. The resulting data was transformed to provide an estimate of plant density/m^2. The same dataset was also transformed to determine the proportion of the total plant number represented by clover seedlings.

Assessment of plant characteristics

A single 20 cm long section of seed drill row was randomly selected within each plot, and eight plants of each sown species (roots and shoots) were removed. Some plots required the selection of more than a one row section due to the low plant density. Any residual soil was removed from the roots using water. The root and shoot length of each plant was measured using the longest root and living leaf (primary stem). This was then transformed to represent the root to shoot ratio for each species. The total leaf area was measured using the program Winfolia (Regent Instruments INC 2006a version) and a flatbed scanner. The trifoliate leaves of the clover plants were removed from the top of the petioles, while the grass leaves were cut 1 cm above the roots. The combined leaf area for the eight plants of each species was assessed. After drying the plants in an oven for 48 hours, the combined dry weight of the eight plants (roots and shoots) was assessed using an analytical balance. These assessments were made monthly for three months, beginning two months after sowing.

4.2 Glasshouse experiment

An experiment was conducted in a glasshouse at the University of Tasmania (42° 54' 6" S / 147° 19' 38" E). This experiment aimed to examine differences in development rate and potential for light interception between red clover cv. Rubitas, Talish clover cv. Permatas, Caucasian clover cv. Kuratas, winter active cocksfoot cv. Uplands, and summer active cocksfoot cv. Megatas. The experiment commenced in December 2017 and concluded in February 2018. The average temperature of the glasshouse during this period was 22 °C (Appendix 2). Irrigation was applied three times daily for 15 mins at 6:45 am, 10 mins at 1 pm, and 6 mins at 4 pm to ensure water availability was not limiting.

4.2.1 Seed germination

Individual plastic containers (length 270 mm x width 195 mm x depth 40 mm) were used to hold 360 seeds of red clover, Talish clover, Caucasian clover, winter active cocksfoot and summer active cocksfoot. The containers were lined with filter paper to which 16 ml of distilled water was added. The containers were placed in a germination cabinet with an average temperature of 20 °C, as this is the base temperature for the germination of most temperate pasture species (Moot *et al.* 2000). The cabinet was set to a cycle of 15 hours of light to 9 hours of darkness. Seeds were left to germinate for 14 days until the majority of seedlings presented cotyledon emergence. Moisture levels inside the containers were monitored during this time, and additional distilled water added when required to maintain a moist environment.

4.2.2 Experimental design

The experiment was conducted in a completely randomised block design with four blocks (**Figure 2**). This design was used to counter any potential effects of the observed light gradient on the bench. Each block contained 45 pots of a 1 L volume with a 14 cm diameter and 15 cm height. Pots were randomised within the blocks weekly, to minimise potential differences in microclimate. The pots were filled with Australian Standard (AS 3743-2003) Premium Grade potting mix to 2 cm below the rim of the pot. The mix was flattened using a 2 cm deep wooden disk to ensure uniformity of volume. Plants were sown as monocultures, with 45 pots allocated to each species. The seedlings were pricked out in pairs into their allocated pots with five pairs placed in a ring at an even spacing from the pot rim and other pairs (**Figure 2**).

Figure 2. Experimental design and single replication.

Seven days after transplanting, any dead seedlings were replaced (

Appendix 3). Two weeks after transplanting, the weaker seedling in each pair was removed leaving five seedlings per pot. Clover seedlings were inoculated one day after transplanting, the group B rhizobia strain was applied to both red clover and Talish clover seedlings, while Caucasian specific rhizobia strain cc283b, was applied to the Caucasian clover seedlings. Rhizobia were applied to the base of seedlings in a spray comprising of a ratio of 45 g of rhizobia to 1 L of distilled water. Pyrethrum was applied over the course of the experiment to control aphid populations present in the glasshouse.

4.2.3 <u>Measurements</u>

Over the course of the experiment several measurements were taken. Leaf number was assessed weekly for each plant, and only fully expanded leaves were counted. One pot containing each species was randomly selected from each block, and the plants were removed including the roots. This began once the majority of plants presented at least one true leaf, this occurred two weeks after transplanting. Any residual potting mix was removed with water. The root and shoot length were measured utilising the longest root and living leaf, this was then transformed to become the root to shoot ratio. The trifoliate clover leaves were cut from the top of the petiole, while the grass leaves were cut 1 cm above the roots. The total leaf area for each plant was then assessed using the program Winfolia (Regent Instruments INC 2006a version) and a flatbed scanner. This methodology was repeated weekly for the following eight weeks. Due to the rate of seedling growth, and pot size, roots from individual grass plants could not be separated after week 6, therefore, the root to shoot ratio could not be assessed.

4.3 Statistical analysis

Q-Q plots were used to determine the normality of the datasets. The datasets included repeated measures, so Mauchly's test of sphericity was utilised, to ensure a mixed model analysis would be appropriate. The correlation structure with the lowest scores for Akaike's Information Criterion (AIC), Hurvich and Tsai's Criterion (AICC), and Schwarz's Bayesian Criterion (BIC) were selected. A diagonal structure was utilised for all datasets except those for clover proportion and individual plant leaf area collected in the glasshouse experiment, where an ARMA (1, 1) was used. Univariate ANOVA analyses was utilised to determine the effects of factors at single time points. Least Significant Difference (LSD) set to $P<0.05$ was used to determine differences between treatments. All statistical analysis was undertaken using the software package SPSS (version 24).

5 Results

5.1 Field experiment

5.1.1 Representation of clover in mixed swards

The clover proportion was determined using plant number. A mixed model analysis highlighted that grass seeding rate had a significant (F=33.247, P<0.01) effect on the clover proportion. In all grass/legume pairings the highest clover proportion was recorded when the lowest grass seeding rate was utilised. At the conclusion of observations there were slight differences in clover proportion between treatments containing the lowest grass seeding rate (1 kg/ha) and the highest grass seeding rate (5 kg/ha) for each clover species 22-39%, 22-44% and 30-31% for Caucasian clover, Talish clover and red clover respectively when sown with cocksfoot. However, these differences were not significant (P>0.05).

Species pairing had a significant (F=4.671, P<0.01) effect on the clover proportion. Successful clover establishment (clover proportions of 20-45%) was achieved for all three clover species utilising different combinations of grass species, and grass seeding rate. Successful establishment was recorded for Talish clover and Caucasian clover when paired with summer active cocksfoot at the grass seeding rate of 3 kg/ha. Clover proportions ranged from 35% to 28%, and 42% to 32%, for these species respectively, at weeks 1 and 12 of observations (**Figure 3**a and **Figure 3**c). It was also achieved when Talish clover or Caucasian clover was paired with winter active cocksfoot at the grass seeding rate of 5 kg/ha, presenting clover proportions that ranged from 45% to 27% and 44% to 27% respectively, at week 1 and week 12 of observations (**Figure 3**b and **Figure 3**d). Successful establishment of red clover was achieved when sown with either cocksfoot species at the grass seeding rate of 5 kg/ha, clover proportions ranged from 39% in week 1 to 29% in week 12 of observations (**Figure 3**e and **Figure 3**f). There was no significant (P>0.05) difference in clover proportion recorded between sowing treatments that contained summer active cocksfoot or winter active cocksfoot.

Results

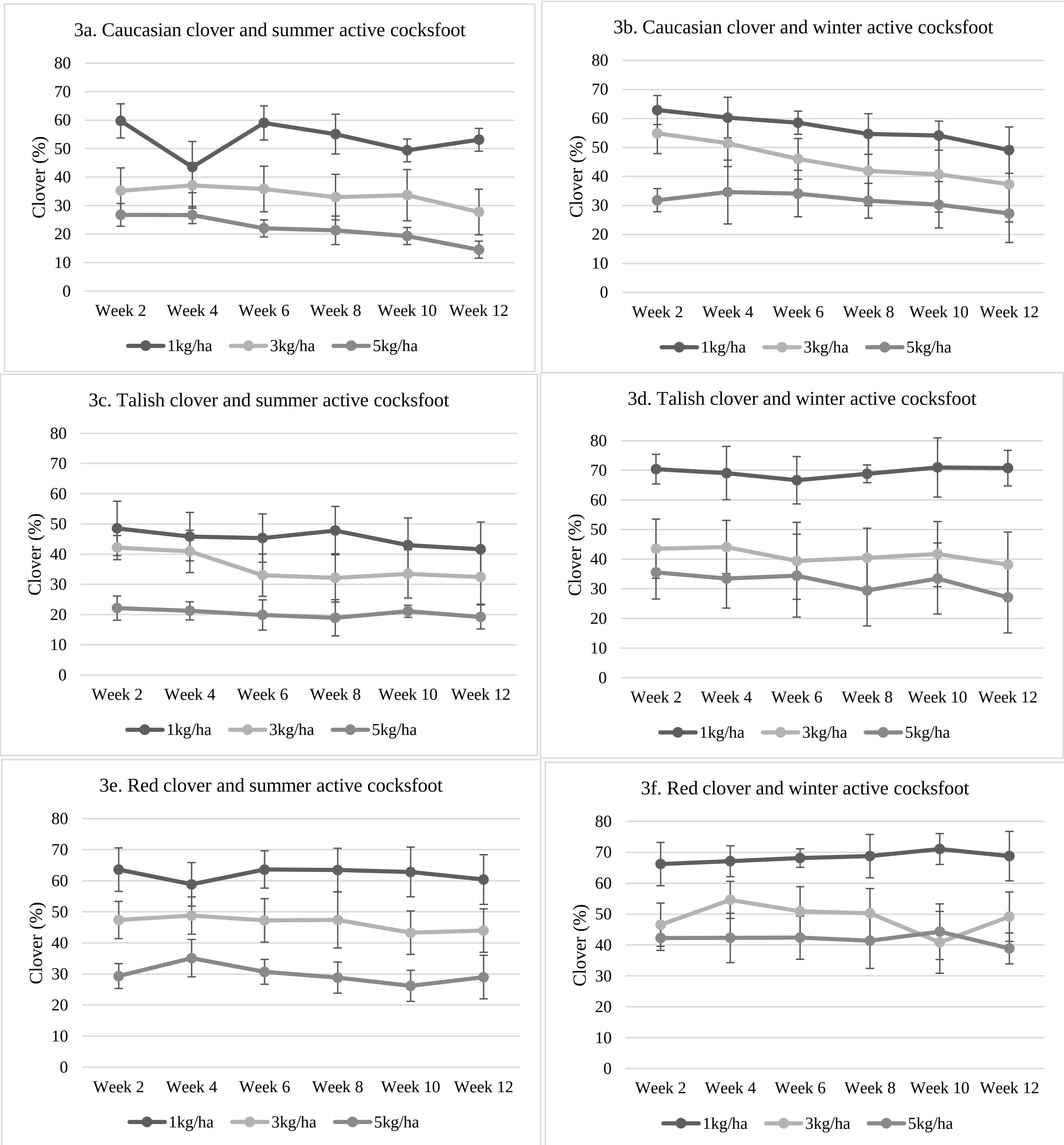

Figure 3. Average proportion of clover present in each seeding treatment at three grass seeding rates. Clover percentage calculated using plant number in a fixed location. Error bars represent ± one SEM.

Time also had a significant (*F*=5.321, *P*<0.01) effect on the clover proportion. Between weeks 1 and 12 the clover proportion recorded in treatments containing Caucasian clover declined by a mean of 10% (**Figure 3**a and **Figure 3**b), however, this decline was not significant (*P*>0.05).

There was no significant (*P*>0.05) interaction observed between grass seeding rate, species pairing or time. A significant (*F*=4.26, *P*<0.01) block effect was recorded.

5.1.2 Plant density

A mixed model analysis highlighted that species pairing (*F*=23.3, *P*<0.01) and grass seeding rate (*F*=130.5, *P*<0.01) had a significant effect on the plant density. A significant (*F*=3.09, *P*<0.01) interaction was observed between these two variables. Univariate ANOVA analysis highlighted plant density to be significantly (*P*<0.05) higher in sowing treatments that contained grass species sown at the seeding rate of 5 kg/ha, with the exception of measurements at week 5 (**Figure 4** and **Figure 5**). There was no significant (*P*>0.05) difference in plant density observed between treatments containing summer active cocksfoot and winter active cocksfoot. A significant (*P*<0.05) decline in the mean number of Caucasian clover seedlings was observed between week 1 and week 12 of observations (**Figure 4** and **Figure 5**). Seedling number declined from a mean of 90 plants/m^2 in week 1, to 64 plants/m^2 in week 6, which is decline of 30%. A significant (*F*=9.6, *P*<0.01) block effect was also observed.

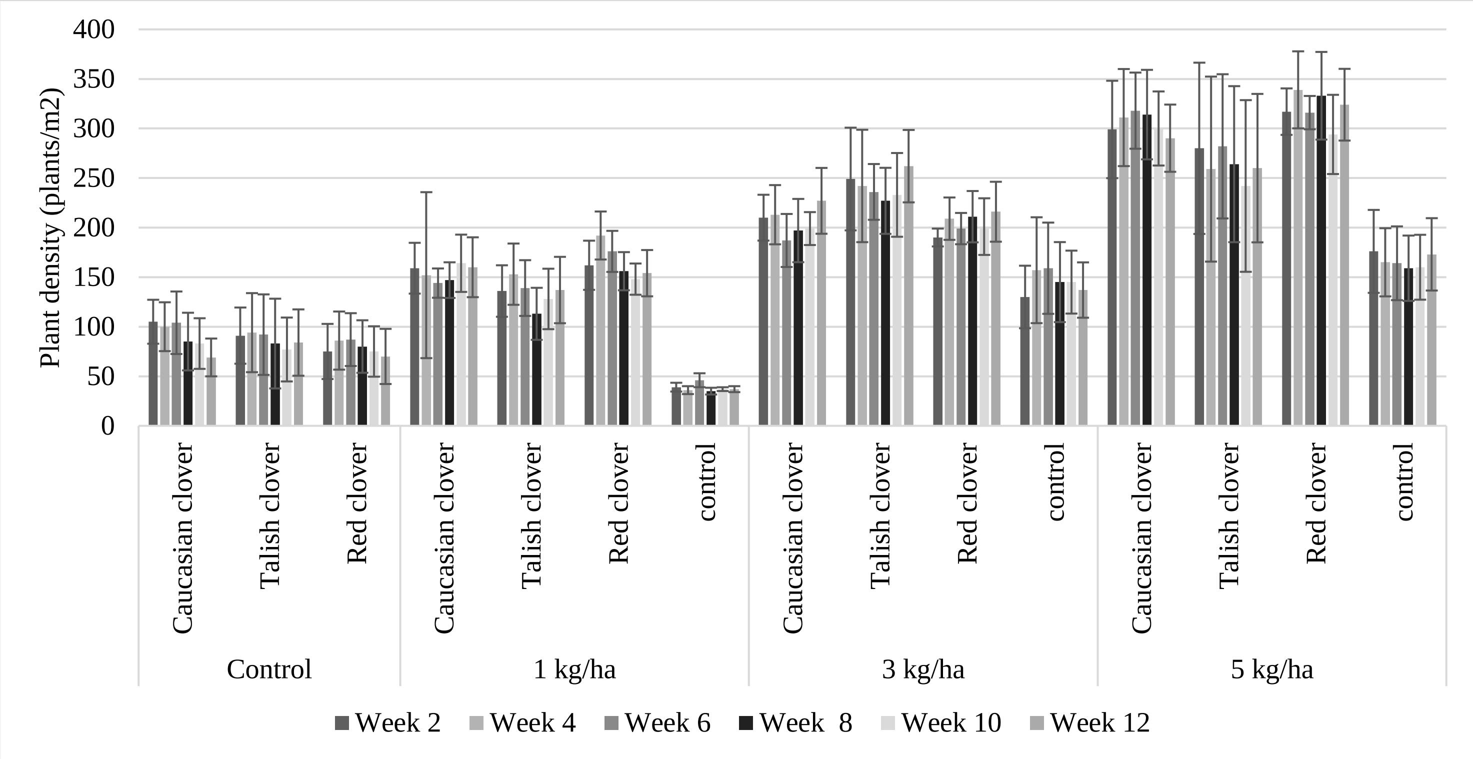

Figure 4. Plant density of sown species (grass and/or clover seedlings) when sown with summer active cocksfoot at three grass seeding rates. Error bars represent ± SEM.

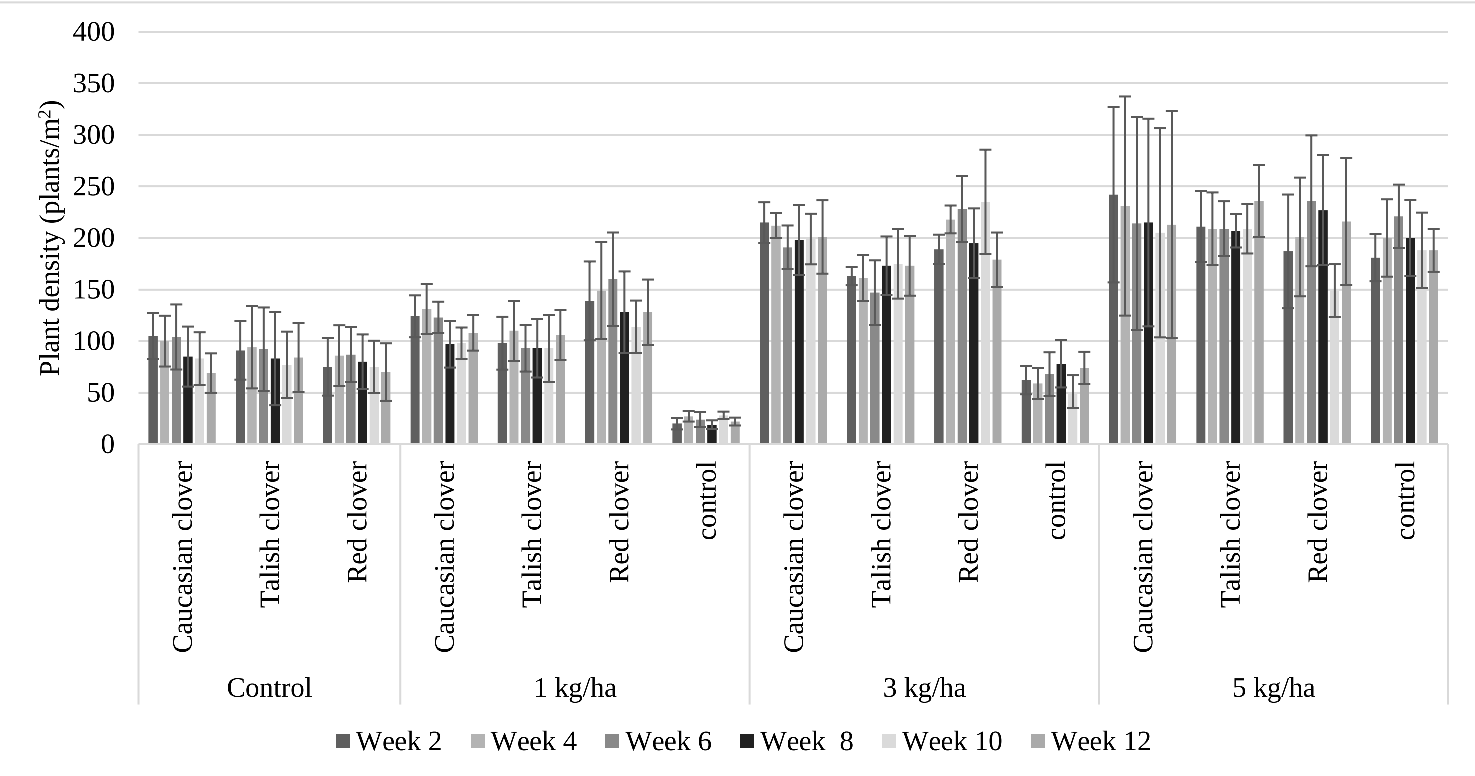

Figure 5. Plant density of sown species (grass and/or clover seedlings) when sown with winter active cocksfoot at three grass seeding rates. Error bars represent ± SEM.

5.1.3 Leaf number

A mixed model analysis highlighted that species (F=38.84, P<0.01) and grass seeding rate (F=3.17, P=0.03) had a significant effect on the mean number of leaves observed. Summer active cocksfoot and winter active cocksfoot presented a faster rate of leaf emergence than Talish clover and Caucasian clover, and presented a significantly (P<0.05) greater number of leaves than these clover species from weeks 1 to 11 (**Figure 6**). Red clover plants displayed a rapid increase in leaf emergence rate at week 6 of observations (**Figure 6**), this resulted in a significantly (P<0.05) greater mean leaf number than Talish clover and Caucasian clover, and a comparable (P>0.05) mean leaf number with winter active cocksfoot from weeks 8 to 11 (**Figure 6**). The increase in leaf emergence rate displayed by red clover correlated with the production of secondary stems. Talish clover and Caucasian clover presented a comparable (P>0.05) mean leaf number from weeks 1 to 11.

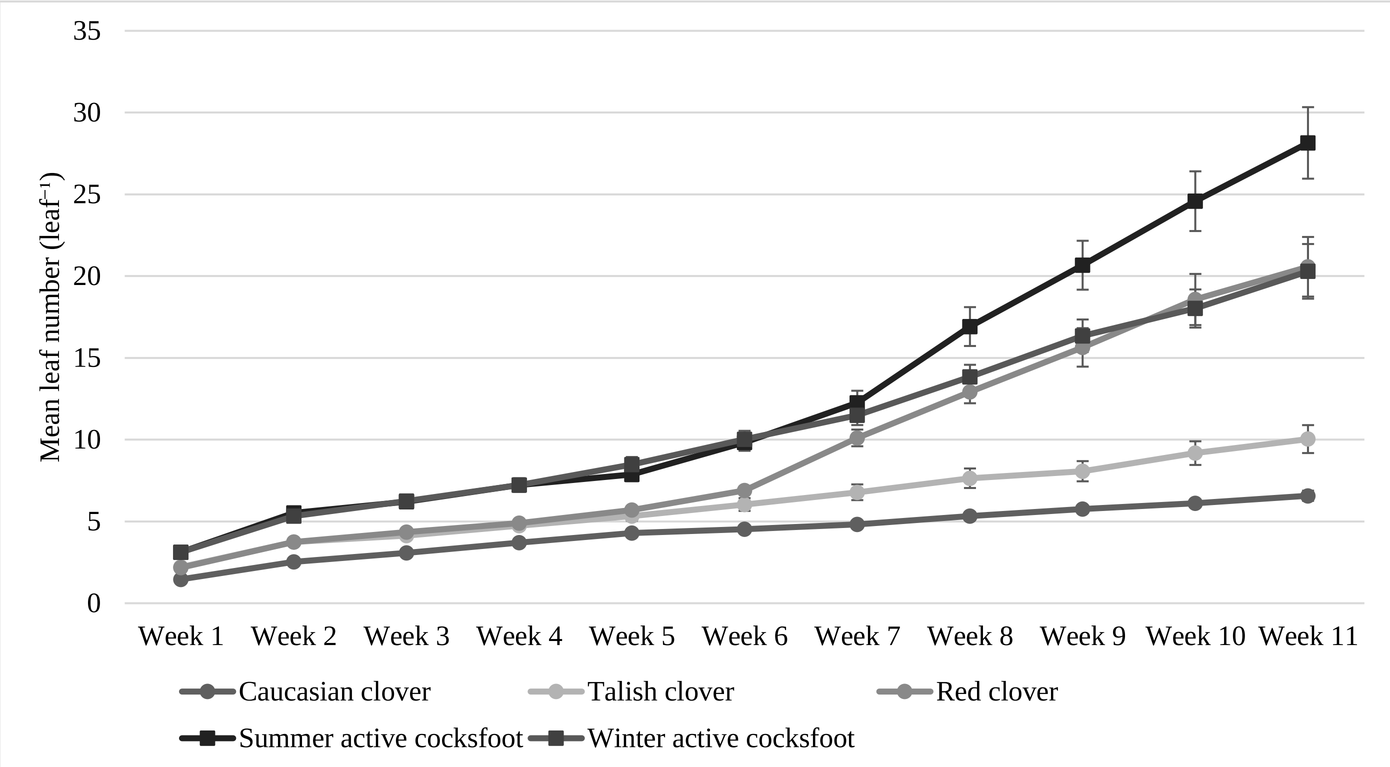

Figure 6. Mean leaf number for pasture seedlings recorded over 11 weeks. Error bars represent ± SEM.

The leaf emergence rate of both cocksfoot species was higher when sown at seeding rates of 5 kg/ha compared to 1 kg/ha, although there was no significant (*P*>0.05) difference in mean leaf number recorded (**Figure 7**). In week 11, summer active cocksfoot seedlings sown at 5 kg/ha had a mean of 33±13.3 leaves, compared to 25±13.3 leaves recorded on plants sown at 1 kg/ha. Similarly, a mean of 23±13.3 leaves was recorded on winter active cocksfoot seedlings sown at 5 kg/ha, compared to 17±13.3 leaves on plants sown at 1 kg/ha.

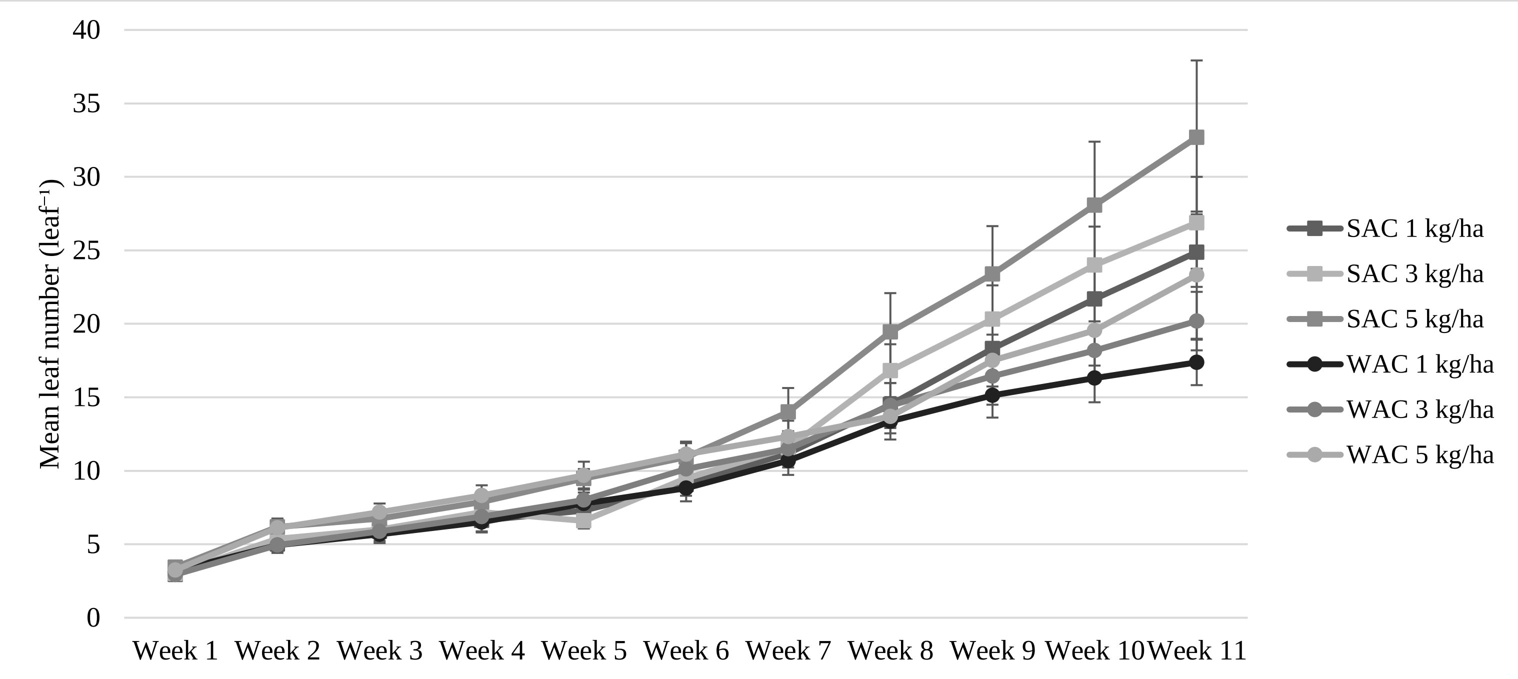

Figure 7. Mean leaf number for summer active cocksfoot (SAC) and winter active cocksfoot (WAC) plants sown at three grass seeding rates. Error bars represent ± SEM.

5.1.4 <u>Leaf area</u>

The combined leaf area was determined for each species utilising eight plants. A mixed model analysis highlighted that species (*F*=60.67, *P*<0.01) and time (*F*=111.03, *P*<0.01) had a significant effect on the combined leaf area, while grass seeding rate and species pairing did not have a significant (*P*>0.05) effect. The two cocksfoot species maintained a significantly (*P*<0.05) greater combined leaf area than Talish clover and Caucasian clover at all sampling points (**Table 4**). The combined leaf area of red clover was significantly (*P*<0.05) greater than all species at each time point, with the exception of summer active cocksfoot in December (**Table 4**). A mixed model analysis undertaken for each species highlighted that there was no significant (*P*>0.05) difference in combined leaf area between seedlings grown in a monoculture or in a grass/ legume pairing.

Table 4. Mean shoot length and root to shoot ratio for individual plants, and the mean combined leaf area of eight plants as recorded at three time points.

Variable	Species	December	January	February
Shoot length (cm)	Caucasian clover	7.5a	8.2a	5.9a
	Talish clover	5.9b	6.9b	5.1a
	Red clover	8.1c	10.2c	8.2b
	Summer active cocksfoot	13.2d	13.6d	12.7c
	Winter active cocksfoot	13.4d	13.4d	12.5c
	SEM	0.2	0.3	0.3
Root to shoot ratio	Caucasian clover	1.3	1.2	1.9
	Talish clover	1.3	1.2	1.7
	Red clover	1.4	1.1	1.6
	Summer active cocksfoot	0.5	0.6	0.7
	Winter active cocksfoot	0.5	0.5	0.7
Leaf area (cm^2)	Caucasian clover	41.2a	42.7a	43.1a
	Talish clover	29.5b	43.4a	60.2a
	Red clover	60.6c	150.6b	274.4b
	Summer active cocksfoot	66.7c	91.3c	163.3c
	Winter active cocksfoot	51.3d	71.5d	105.9d
	SEM	2.2	5.9	12.1

Means within columns with different letters are significantly different (P=0.05), determined utilising standard error of the mean (SEM).

5.1.5 Root to shoot ratio

Root to shoot ratios were calculated utilising root and shoot length, and differed greatly between the grass and clover species. Root to shoot ratios favouring shoot growth, of between 0.5 and 0.7 were recorded for summer active and winter active cocksfoot between December and February (**Table 4**). In contrast, root to shoot ratios recorded for the clover species were greater than 1, favouring root length. Root to shoot ratios increased for all clover species over the three sampling points. The largest increase in root to shoot ratio from 1.3 in December to 1.9 in February was recorded on Caucasian clover plants (**Table 4**).

5.1.6 Shoot length

A mixed model analysis displayed species (F=237.5, P<0.01) and grass seeding rate (F=2.72, P=0.04) to have a significant effect on mean shoot length. Species pairing was not recorded to have significant (P>0.05) effect. Univariate ANOVA analyses highlighted that both cocksfoot species presented significantly (P<0.05) greater mean shoot lengths than the three clover species at all sampling points (**Table 4**). While, red clover displayed a significantly (P<0.05) greater mean shoot length than Talish and Caucasian clover at all observations (**Table 4**).

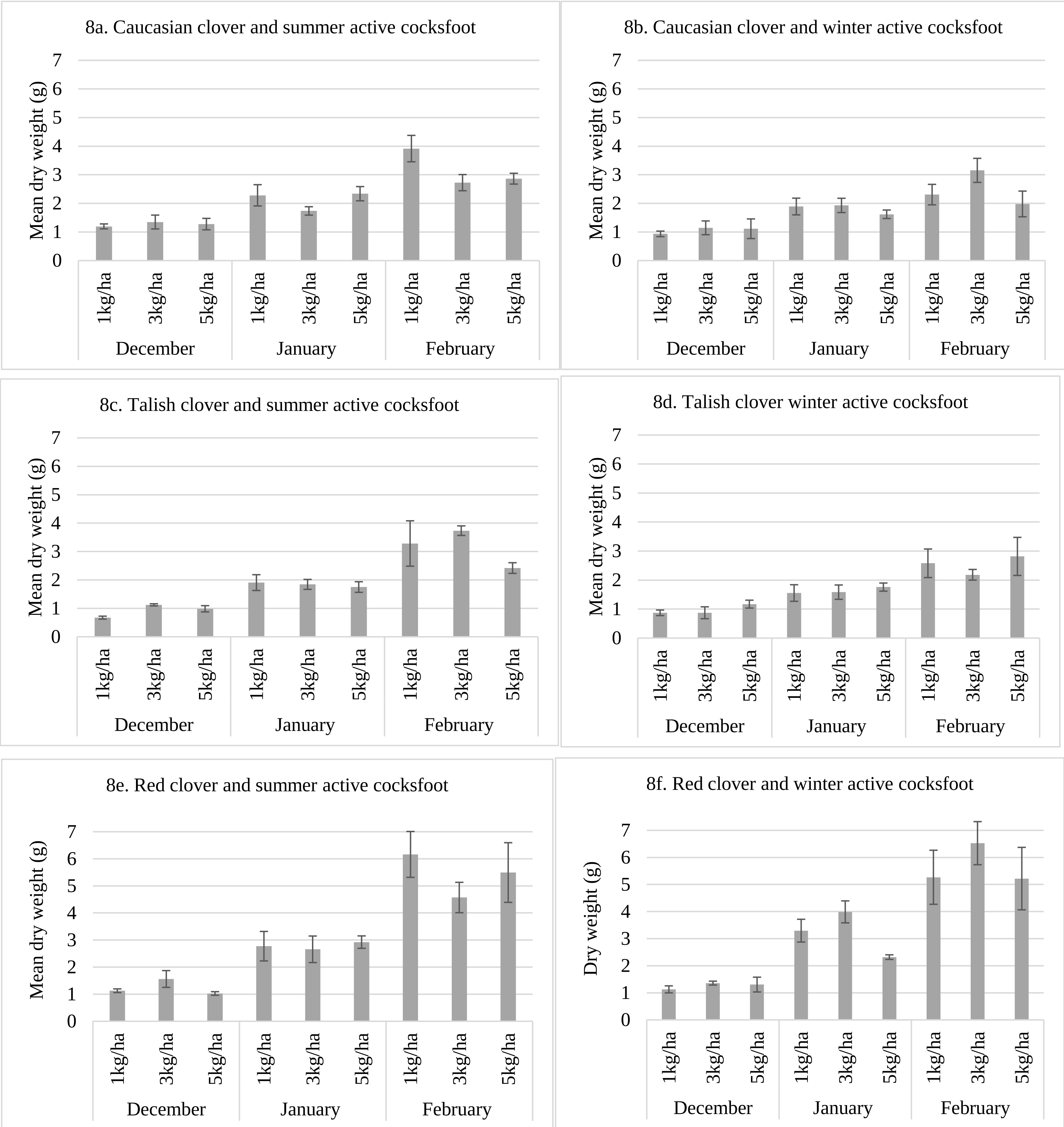

Figure 8. Combined whole plant dry weight (g) of eight grass plants and eight clover plants in each sowing treatment and three grass seeding rates. Error bars represent ±SEM.

5.1.7 Dry weight

A mixed model analysis reflected that species pairing had a significant (F= 10.99, P<0.01) effect on the combined dry weights recorded, while grass seeding rate did not (F= 1.464, P=0.23). As expected the largest combined dry weights were recorded at the final observation in February (**Figure 8**). In February there were some differences in mean biomass recorded between sowing treatments at 2.8 g, 2.8 g and 5.5 g for treatments containing Caucasian clover, Talish clover and red clover respectively, however this difference was not significant (P>0.05). There was no significant (P>0.05) difference recorded between treatments that contained summer active cocksfoot or winter active cocksfoot at 3.9 g and 3.5 g respectively (**Figure 8**). A mixed model analysis undertaken for each species highlighted that there was no significant (P> 0.05) difference in the combined dry weights recorded for plants grown in a monoculture or in a grass/ legume pairing.

5.2 Glasshouse experiment

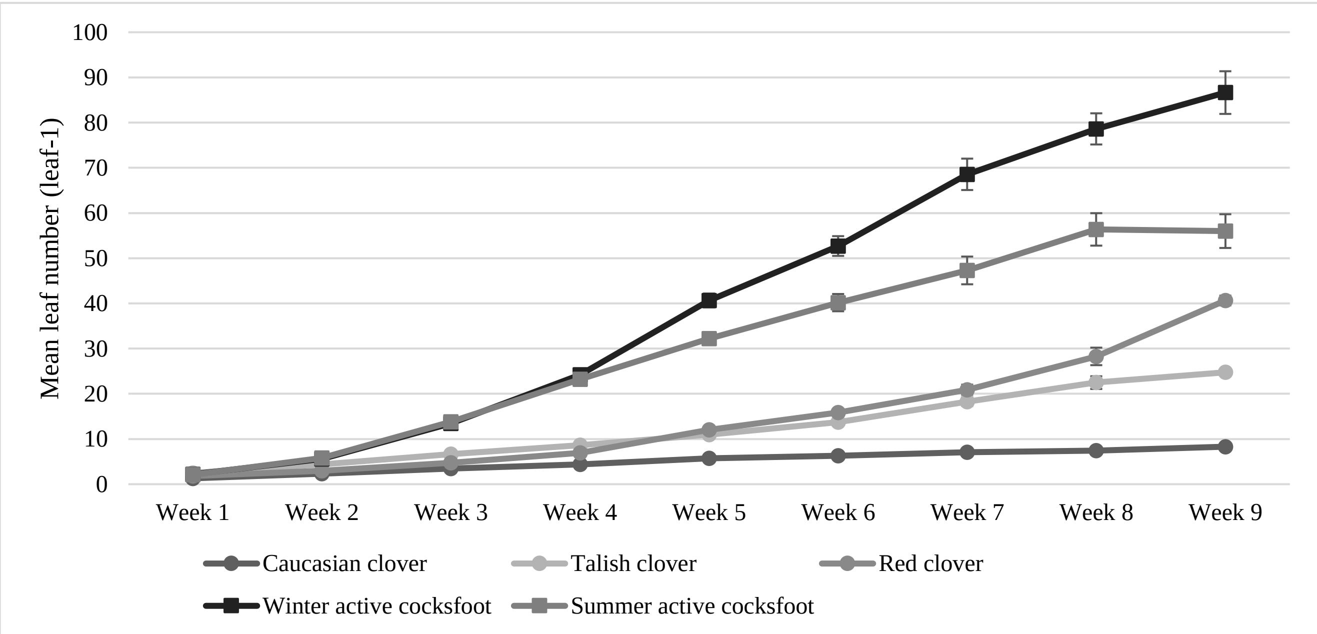

Figure 9. Mean leaf number for five pasture species recorded weekly for nine weeks. Error bars represent ±SEM.

5.2.1 Leaf number

Mixed model analysis reflected that species had a significant (F= 5.29, P= 0.01) effect on the mean leaf number. Both cocksfoot species accumulated leaves at a faster rate than clover species (**Figure 9**) and maintained a significantly (P<0.05) higher mean leaf number in all weeks with the exception of data recorded in weeks 1 and 9. Caucasian clover presented a significantly (P<0.05) lower mean leaf number than all other species from week 1 to week 8 of observations (**Figure 9** and **Figure 10**). Red clover displayed an exponential increase in leaf emergence rate at week 6, this correlated with the observation of secondary stems on this species. The mean leaf number recorded for red clover became comparable (P>0.05) with summer active cocksfoot in week 9.

Figure 10. Comparison of leaf number presented by of Caucasian clover (top), red clover (right) and Talish clover (bottom) at week 8 of observations.

5.2.2 Leaf area

A mixed model analysis highlighted that species had a significant (F=30.4, P<0.01) effect on leaf area. Univariate ANOVA analyses highlighted that summer active cocksfoot displayed a significantly (P<0.05) greater leaf area than all clover species from week 1 to week 9 at 4.8-318.3 cm^2 compared to 1.9–89.4 cm^2, 3.1-117.2 cm^2 and 2.9-251.7 cm^2 for Caucasian clover, Talish clover and red clover respectively (**Table 5**). The three clover species maintained a comparable (P>0.05) leaf area from week 1 to week 5 at 1.9-41.5 cm^2, 3.1-54.8 cm^2 and 2.9-64.6 cm^2 for Caucasian clover, Talish clover and red clover. However, from weeks 6 to 9 red clover displayed a significantly (P<0.05) greater leaf area than Talish clover and Caucasian clover at 113.4-251.7 cm^2 compared to 78.6-117.2 cm^2 and 54.3-82.4 cm^2 (**Table 5**) This correlated with the observation of secondary stems on this species, and resulted in a comparable (P>0.05) leaf area with winter active cocksfoot in week 9.

Table 5. Mean leaf area, shoot length and root to shoot ratio for individual plants of Caucasian clover (CC), Talish clover (TC), red clover (RC), summer active cocksfoot (SAC) and winter active cocksfoot (WAC).

Variable	Species	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	Wk 9
Leaf area (cm^2)	CC	1.9a	6.0a	16.7a	20.8a	41.5a	54.3a	79.3a	75.1a	82.4a
	TC	3.1b	6.3a	17.9a	46.0b	54.8a	78.6ab	102.7a	121.0a	117.2a
	RC	2.9b	6.9a	22.0a	43.5b	64.6a	113.4b	149.9b	196.8b	251.7b
	WAC	2.7b	6.9a	32.8b	99.9c	154.4b	181.8c	230.5c	282.0c	254.3b
	SAC	4.8c	14.3b	48.1c	144.3d	157.6b	256.3d	272.1d	309.6c	318.3c
	SEM	0.2	0.4	1.5	5.7	7.1	9.3	9.7	12.3	11.9
Shoot length (cm)	CC	NA	7.2a	12.8a	13.0a	18.9a	19.9a	22.4a	21.2a	23.3a
	TC	NA	6.0a	10.1b	13.3a	15.3b	18.3a	18.8b	19.2a	17.6b
	RC	NA	7.7a	13.0a	18.5b	18.1ab	20.6a	21.1ab	21.7a	20.1ab
	WAC	NA	11.9b	21.3c	29.2c	32.3c	33.6b	36.8c	39.0b	41.2c
	SAC	NA	17.1c	26.3d	39.0d	43.2d	50.4c	47.6d	47.1c	47.5d
	SEM	NA	0.5	0.7	1.1	1.2	1.3	1.2	1.3	1.3
Root to shoot ratio	CC	NA	1.6	1.6	1.3	1.2	1	0.8	0.8	0.7
	TC	NA	2.1	1.6	1.4	1.4	1.3	1.1	1.1	1.1
	RC	NA	1.8	2	1.3	1.4	1.5	1.2	1.4	1.3
	WAC	NA	0.9	0.9	0.9	0.9	0.8	NA	NA	NA
	SAC	NA	0.8	0.7	0.7	0.6	0.7	NA	NA	NA

Means within columns with different letters are significantly different (P=0.05), determined utilising standard error of the mean (SEM).

5.2.3 Root to shoot ratio

Differences in root to shoot ratio were observed between the grass and the clover species. The two cocksfoot species maintained root to shoot ratios which favoured shoot length, at 0.6 -0.8 for summer active cocksfoot and 0.8-0.9 for winter active cocksfoot (**Table 5**). The root to shoot ratios for the three clover species were observed to decline from week 2 to week 9 of observations. Declining from 2.1 to 1.1, 2 to 1.2, and 1.6 to 0.7 for Talish clover, red clover and Caucasian clover respectively (**Table 5**).

5.2.4 Shoot length

A mixed model analysis highlighted that species (F=651.4, P<0.05) and time (F=433.7, P<0.05) had a significant effect on mean shoot length. A significant (F= 19.3, P<0.01) interaction was recorded between these two variables. The mean shoot length for summer active cocksfoot was significantly (P<0.05) greater than winter active cocksfoot from week 2 to 9 at 17.1-47.5 cm and 11.9-41.2 cm respectively (**Table 5**). Both cocksfoot species presented a significantly (P<0.05) greater mean shoot length than Caucasian clover, Talish clover and red clover at 7.2-23.3 cm, 6-17.6 cm and 7.7-20.1 cm from weeks 2 to 9 (**Table 5**). A significant (F=2.9, P=0.03) block effect was also observed.

6 Discussion

6.1 Establishment of Caucasian clover and Talish clover

Successful establishment of both Talish clover and Caucasian clover was achieved utilising several combinations of grass seeding rate and grass species. Successful establishment in this experiment was classified as maintaining the recommended proportion of legumes in the sward, at 20-45% (Thomas 1992), for the duration of observations. For both clover species this was achieved when paired with summer active cocksfoot or winter active cocksfoot, at grass seeding rates that fell within the breeders recommended range of 2-5 kg/ha (Hall and Hurst 2012c; Hall and Hurst 2012b). Previous studies have also investigated the effect of grass seeding rate on clover establishment. However, the short length of the current experiment, and a difference in the methodology used to determine clover proportion, makes a direct comparison of the results difficult. In the current study plant number was used to determine clover proportion, while other studies have commonly utilised shoot biomass to determine the same objective. The method utilised in this study does not account for differences in plant size, which is a determining factor when assessing biomass. Clover proportion was assessed using plant number, rather than shoot biomass, due to the young age of seedlings in this experiment.

Successful establishment was achieved for Talish clover and Caucasian clover when paired with winter active cocksfoot at the grass seeding rates of 3 kg/ha and 5 kg/ha. This result may indicate an improved compatibility with winter active cocksfoot, as successful establishment was not recorded for either clover species when sown with summer active cocksfoot at the grass seeding rate of 5 kg/ha. However, the density of grass plants was lower in sowing treatments that contained this species, despite the same initial grass seeding rates. This may have created a more favourable environment for clover seedling growth with lower competition experienced for resources (Tow and Lazenby 2001). This difference may have also been due to the difference in seasonal activity between the two grass species. Summer active cocksfoot produces the majority of its biomass over the spring/ summer period as allowed by soil moisture availability. In contrast, winter active cocksfoot produces the greatest proportion of its biomass over the autumn to spring seasons. Plants then enter a state of dormancy at the end of spring/ early summer as the amount of available soil moisture declines (Hackney and Dear 2007). This increases the tolerance of winter active cocksfoot to drought, but decreases their productivity level over the summer (Norton *et al.* 2008). This experiment was conducted over the spring/ summer months when this species is at its lowest level of productivity. Further observations should be made outside of the summer season to determine any potential effects on the clover proportion.

Successful establishment of Talish clover and Caucasian clover was achieved when paired with summer active cocksfoot at the grass seeding rate of 3 kg/ha. A decline in the mean seedling number of Caucasian clover and Talish clover seedlings between week 1 and week 12 was recorded in all sowing treatments that also contained summer active cocksfoot. This reduction in seedling number resulted in a decline in the overall mean clover proportion. When paired with summer active cocksfoot at the grass seeding rate of 5 kg/ha, the decline in clover proportion resulted in clover proportions below the recommended 20%, indicating a grass seeding rate less than 5 kg/ha should be selected to facilitate successful establishment. The reduction in seedling number was observed to a greater extent in sowing treatments that contained Caucasian clover. Between week 1 and week 12 the number of Caucasian clover seedlings declined by a mean of 30%, which was inclusive of sowing treatments where this species was sown as a monoculture. This highlights the poor establishment of this species regardless of species pairing, and highlights an increased likelihood that this trend could continue over further weeks. As was observed in a similar study undertaken by Moss *et al.* (1996) when the proportion of Caucasian clover biomass in cocksfoot cv. Grasslands Kara swards was initially reported at over 20% of the total biomass, but gradually declined to only 10% of the total biomass at 16 months after sowing. However, due to the short duration of this experiment, and the noted decline in clover proportion, further observations should be made at the end of the yearlong establishment phase to determine if this conclusion remains valid. These results indicate that hypothesis (1) should be accepted, as successful establishment of Talish clover and Caucasian clover was achieved when these species were paired with cocksfoot at a lowered (3 kg/ha) grass seeding rate.

6.2 Establishment of red clover

Successful establishment of red clover was also achieved when sown with either cocksfoot species, but at the higher grass seeding rate of 5 kg/ha. The decline in seedling number observed in sowing treatments that contained Caucasian clover and Talish clover was not observed in treatments that contained red clover. The number of red clover seedlings displayed little variation, which may indicate a greater ability of this species to compete for resources in the sward. Red clover has been reported to be fast growing during seedling development (Annicchiarico *et al.* 2015), in contrast both Talish clover and Caucasian clover have been described as slow growing during the same development stage (Dodd and Orr 1995; Mills *et al.* 2015). The results of this experiment supported these pervious findings. Red clover seedlings presented a faster rate of leaf development, greater mean leaf area and earlier development of secondary stems, than Talish clover and Caucasian clover seedlings. The combination of these traits indicates a stronger ability to compete for light in the sward, and offers a potential explanation for the improved persistence of red clover seedlings and higher clover proportions in sowing treatments that contained this species. Hurst *et al.* (2000) proposed the rapid germination and seedling growth of red clover plants as an explanation of their improved establishment in perennial ryegrass swards, when compared to the poor establishment of Caucasian clover seedlings under the same conditions.

6.3 Plant Dry Weight and Plant Density

The plant density was higher in sowing treatments with higher grass seeding rates, and in sowing treatments that contained multiple sown species. Previous studies have highlighted that high plant densities lead to increased competition for resources (Langer 1990), and seedlings of a smaller size than those grown in lower plant densities (Lee *et al.* 2017). In response to these findings it was hypothesised (2) that both grass seeding rate and species pairing would affect the plant dry weights. However, the results of this experiment indicate that this hypothesis should be rejected, as there was no significant difference recorded between the combined dry weights in sowing treatments that contained different grass seeding rates, or between seedlings grown in a monoculture or grass/legume pairing. It is possible that the short window allowed for growth in this experiment, meant that seedlings did not develop to a point at which competition for resources significantly affected seedling size. The results also indicate that by lowering the grass seeding rate producers will compromise biomass availability, as with fewer seedlings of the same size and weight a lower plant number would equate to a lower available biomass. However, Black *et al.* (2006b) observed differences of less than 2 t/DM/ha between sowing treatments that contained white clover sown with perennial ryegrass at the grass seeding rates of 3.5 kg/ha, 8 kg/ha and 13 kg/ha, 12 months after sowing. Similarly, Hurst *et al.* (2000) recorded no significant difference in total biomass between mixed swards that contained perennial ryegrass sown at 3.5 kg/ha or 8 kg/ha, 16 months after sowing. The results of these studies indicate that as seedlings develop, the lower plant density may facilitate an improved performance of individual plants, which compensates for the lower plant density. It is possible that the same result may be observed at the current experimental site later in the establishment phase.

The mean combined dry weights were effected by species pairing. Sowing treatments that contained red clover presented significantly (P<0.05) higher combined dry weights at all sampling points, than those that contained Talish clover or Caucasian clover, indicating the greater dry weight of red clover seedlings. This is in agreement with a study undertaken by Hurst *et al.* (2000) who observed red clover seedlings to present a greater total plant dry weight than Caucasian clover seedlings at 43 and 83 days after sowing. Red clover was the only clover species to develop secondary stems in the field experiment, and the rapid accumulation of leaves and stolons that occurred during secondary development, help to explain the greater recorded dry weights. These results suggest that producers should select red clover over Talish clover or Caucasian clover when considering a comparison of biomass production, however, in selecting red clover there would be a compromise in drought tolerance and persistence. The combined dry weights were lower in sowing treatments that contained winter active cocksfoot, in comparison to those containing summer active cocksfoot although this was not significant. This difference in biomass production is in agreement with previous studies by Kiaer *et al.* (2013) and Culvenor *et al.* (2016) who attributed the lower biomass production to the increased drought tolerance of winter active cocksfoot species, due to their fine leaf morphology.

6.4 Individual seedling characteristics

6.4.1 Light interception: Leaf area and leaf emergence rate

Several plant characteristics related to light interception were assessed on seedlings grown in the field and in the glasshouse. Differences between species in their rate of leaf development, leaf area, and shoot length were examined. It was important to examine these characteristics in an environment where water and nutrients were not limiting as when plants are exposed to conditions of low nutrient or water availability, a greater investment is placed into root growth at the cost of shoot development (Kiaer *et al.* 2013; Tow and Lazenby 2001). There is natural variation in water and nutrient availability across a paddock, which could result in observable differences in shoot development due to environment rather than to species.

Discussion

Clover species grown in the glasshouse maintained a comparable leaf area from week 1 to week 6 despite differing significantly in leaf number. Caucasian clover maintained a significantly (P<0.05) lower leaf number than Talish clover and red clover, but maintained a comparable leaf area. This indicates that the leaves of Caucasian clover were of a greater individual size than that of red clover or Talish clover. This observation is in agreement with a study under taken by Black and O'Kiely (2007) who also observed a comparable leaf area between plants of several Caucasian clover and red clover cultivars, despite red clover plants presenting a significantly greater leaf number. Caucasian clover has also been observed to maintain a comparable leaf area with white clover, despite a threefold difference in leaf number (Black *et al.* 2006a). No previous studies investigating leaf area or leaf appearance rate for Talish clover have been published so no comparison can be made. These results indicate a similar potential for light interception in terms of plant leaf area between the three species during primary seedling development.

The initiation of secondary stems on clover plants had a significant effect on the rate of leaf emergence and leaf area. Secondary stems were observed on red clover plants in week 6 and on Talish clover plants in week 8 of observations in the glasshouse experiment, and not observed at all on Caucasian clover plants. Red clover was the only species to present secondary stem initiation in the field experiment. The development of secondary stems on red clover seedlings correlated with an exponential increase in leaf number, which translated to a rapid increase in leaf area. In the glasshouse experiment this increase in leaf emergence rate resulted in a comparable leaf area with winter active cocksfoot at the conclusion of observations. Observations of leaf area and leaf emergence in the field experiment began at a later stage of seedling development and spanned a greater length of time. The initiation of secondary stems on red clover seedling in the field was observed in week 6 of observations (12 weeks after sowing), which correlated with the first recorded measurement of leaf area in December, when red clover plants presented a greater leaf area than all species except summer active cocksfoot. Later observations displayed red clover to have greater leaf area than all other species. The development of secondary stems (stolons) by red clover also resulted in seedlings covering a greater area horizontally and vertically in the canopy. This would have further increased the ability of red clover to compete for, and intercept light as seedlings could place stolons in the spaces between seed rows to access light without interference from other seedlings. These results highlight the improved potential for light interception provided by the development of secondary stems, and highlights a significant difference in potential for light interception between red clover, Caucasian clover and Talish clover.

Previous experiments have quantified this difference in secondary stem development between pasture species by observing differences in their requirement for heat units or degree days (°Cd). An experiment undertaken by Black and O'Kiely (2007) recorded the mean degree day requirement for secondary stem initiation on red clover plants at 623 °Cd, to be greater than Caucasian clover, which was not observed to develop secondary stems within the time constraints of the experiment at greater than 670 °Cd. Black *et al.* (2006a) reported Caucasian clover seedlings to require 1180 °Cd for secondary stem initiation, which was considerably greater than that of white clover at 430 °Cd. These results support the observations made in the current study, and offer an explanation for the difference in the timing of secondary stem initiation between the clover species. There have been no published studies presenting the requirements of Talish clover for heat units, so no comparison can be made. The comparatively slow development of secondary stems by Talish clover and Caucasian clover indicates a lesser potential to intercept and compete for light in the sward, compared to that of red clover.

The two cocksfoot species were observed to develop leaves at a faster rate than the three clover species in both the glasshouse and the field experiments. The two cocksfoot cultivars included in this experiment, cv. Megatas (summer active) and cv. Uplands (winter active), were bred for increased tillering (Turner *et al.* 2007) so the rapid accumulation of leaves was as expected. Elevated grass seeding rates appeared to have a positive effect on the leaf emergence rate of the two cocksfoot species, with plants presenting a slightly higher leaf number when sown at higher grass seeding rates. The rapid leaf emergence rate displayed by the two cocksfoot species translated to a greater leaf area, relative to that of Caucasian clover and Talish clover. Previous studies have also observed the lower leaf area of Caucasian clover seedlings relative to other grass species, such as perennial ryegrass (Black *et al.* 2006a), and grazing brome (*Bromus biebersteinii*) (Walker and King 2009) during establishment. The lesser leaf area and leaf development rate of Talish clover and Caucasian clover indicates a poorer ability to intercept, and compete for light than the two cocksfoot species grasses.

6.4.2 Light interception: Shoot length

Shoot length denoted the height of leaves within the canopy. Seedling that extend their leaves to the top of the canopy are able to intercept a greater proportion of the available light in the sward (Stern and Donald 1962a). Shoot length of clover plants was obtained from primary leaves rather than those on secondary stems. Grass plants cultivated in both the glasshouse and the field, presented a greater mean shoot length than the three clover species at all observations indicating a greater potential for light interception in the sward. Comparable shoot lengths were recorded for the three clover species when cultivated in the glasshouse, while significant differences where observed between the three species in the field. This difference in mean shoot length between species cultivated in the field may be due to the difference in competition pressure for light interception. Seedlings grown in the glasshouse were exposed to competition for light from the other seedlings grown in the same pot that were all of a similar height and spaced at a low plant density, while clover seedlings in the field were exposed to competition pressure from grass seedlings and weeds that presented a greater shoot length. Walker and King (2009) reported the shoot length of Caucasian clover to be significantly greater when paired with meadow brome, than when cultivated as a monoculture. This indicates that red clover may place a greater investment into shoot length when placed under competition pressure than Talish clover or Caucasian clover thereby increasing its potential for light interception.

The combination of a significantly lower leaf area, leaf emergence rate and mean shoot length observed on Caucasian clover and Talish clover seedlings, in comparison to the two cocksfoot species, highlights a lesser potential for light interception. This was quantified in an experiment undertaken by Stern and Donald (1962b) who reported that the tendency of grass plants to place their leaves higher in the canopy resulted in these plants intercepting a greater proportion of the light available in the sward, than clover plants with leaves placed lower in the canopy. The observed decline in Caucasian clover seedling number over the duration of the experiment may be partly explained by this limited access to light in the sward. These results suggest that hypothesis (3) should not be rejected, as significant differences between the five pasture species in their rate of leaf emergence, leaf area and shoot length have been determined, which influence the ability of seedlings to intercept light. It suggested that these observed differences resulted in the change in seedling number recorded over the 12 weeks of observations.

6.4.3 Root to shoot ratio

In this experiment root to shoot ratio was determined utilising root and shoot length, other studies have determined the same objective utilising a comparison of root and shoot biomass. It is acknowledged that the results obtained in this study provide only an indication of biomass investment between the roots and shoots. In both the glasshouse and field experiments the two cocksfoot species displayed a greater shoot length comparable to root length, indicating a bias toward shoot growth. Other studies have reported similar bias toward shoot growth, as determined utilising a comparison of biomass, in seedling of other grass species such as perennial ryegrass and meadow brome (Walker and King 2009; Black *et al.* 2006b). This indicates grasses in general, tend to make a greater investment in shoot development during seedling growth.

Caucasian clover, Talish clover and red clover displayed a greater shoot length comparable to shoot length. Root to shoot ratios were lower for clover seedlings cultivated in the glasshouse relative to those recorded in the field experiment. This was as expected as the availability of resources (water and nutrients) would have been lower in the field than in the glasshouse and when resources are limited plants are reported to preferentially allocate resources to the roots to maximise the potential to assess these resources (Tow and Lazenby 2001; Kiaer *et al.* 2013). Root length for plants in the glasshouse were also limited by the depth of the pots with roots of all clover species reaching the base of the pots by week 7 of observations. The development of roots by grass seedlings was also restricted by pot size. By week 7 of observations both cocksfoot species had developed root systems that had expanded to fill the entirety of the pots. In weeks 8 and 9 some grass seedlings were exhibiting symptoms of moisture stress as the irrigation applied was no longer possible for the water to penetrate through the dense root systems. This experiment should be repeated under conditions where pot size is not limiting to better observe the observed change in root to shoot ratio as seedlings develop.

Previous studies have reported results for root to shoot ratio that are in agreement with those observed in the field experiment. Widdup *et al.* (1998) observed up to a threefold greater investment by Caucasian clover into root biomass, comparable to shoot biomass, at 12 months after sowing. It could be argued that this preferential allocation of resources to the roots during seedling development is a central factor in the slow shoot development by this species, and partially responsible for the species poor performance during establishment

(Black *et al.* 2006b). The same might be argued for Talish clover, which also develops a substantial taproot system (Dodd and Orr 1995). However, it is the development of this taproot that conveys the high drought tolerance and persistence of these species.

6.5 Implications for cocksfoot

The slow development rate of cocksfoot during early seedling growth makes it a suitable companion species for Talish clover, Caucasian clover and red clover. Grass seeding rates for summer active and winter active species of cocksfoot can be modified within the breeders recommended rates to allow the successful establishment of companion clover species. However, in applying this management technique, producers will reduce the biomass contribution by grass plants in the sward and decrease the total productivity of the pasture. The results of this study highlighted that winter active cocksfoot may be more compatible with Talish clover and Caucasian clover, compared to summer active cocksfoot. Sowing treatments that contained winter active cocksfoot presented a slightly higher proportion of legumes, and successful establishment was achieved using the highest grass seeding rate (5 kg/ha). This may be explained by the slower rate of leaf emergence, smaller leaf area and shoot length of winter active cocksfoot compared to summer active cocksfoot, indicating a lesser competitive ability for light. Winter active cocksfoot is also reported to have a greater drought tolerance than summer active cocksfoot species, due to its ability to enter a state of dormancy during summer when moisture availability is low (Norton *et al.* 2008). This indicates a greater suitability of this species to the low rainfall areas of the Tasmanian midlands. However, there was a slight difference in biomass production between the species, indicating that when in areas with higher rainfall or access to irrigation summer active cocksfoot should be selected.

6.6 Implications for red clover

Successful establishment of Red clover was achieved with both species of cocksfoot. This study confirmed the results of others, highlighting the ability of this species to be competitive with grasses for light and quick to establish (Annicchiarico *et al.* 2015; Black *et al.* 2009). The recommended legume proportion of between 20-45% (Thomas 1992) was achieved when sown with grasses at the grass seeding rate of 5 kg/ha. The high competitive ability of this species meant that clover proportions well above the recommended range were recorded when grass seeding rates of 1 kg/ha or 3 kg/ha were utilised. Red clover was observed to produce the highest dry weight of the three clover species, indicating that this species is the most productive during establishment. The cultivar utilised in this experiment, cv. Rubitas, has been bred for greater drought tolerance and to be prostrate in growth habit, improving its persistence under moisture stress and grazing (Nichols *et al.* 2012). These traits make this cultivar better suited to the dryland and low rainfall conditions of the Tasmanian midlands than previously available cultivars.

6.7 Implications for Caucasian clover

The results of this study highlighted that successful establishment of Caucasian clover can be achieved when paired with summer active or winter active cocksfoot species at lowered grass seeding rates. This was achieved when Caucasian clover was paired with winter active cocksfoot at 3 kg/ha and 5 kg/ha, and when paired with summer active cocksfoot at 3 kg/ha. The combination of a lowered grass seeding rate and pairing with a slow growing grass species overcame the issues of poor establishment of Caucasian clover recorded in previous studies (Hurst *et al.* 2000; Black *et al.* 2006b; Mills *et al.* 2015). However, this experiment covered only a short window of the pasture establishment phase that commonly extends to include the first year after sowing. A decline in the number of Caucasian clover seedlings was observed in all sowing treatments, including those where it was sown as a monoculture. There is potential that this observed decline could continue over the remainder of the year, resulting in unsuccessful establishment in the long term. Caucasian clover presented a lower dry weight than that recorded for red clover, indicating a lower productivity during establishment, as was observed in previous studies (Hurst *et al.* 2000; Black *et al.* 2006b). To obtain the benefits of high drought tolerance and persistence attributed to Caucasian clover, producers will have to compromise biomass availability for at least the first three years, due to the slow early growth of this species (Seguin 2007). Therefore, use of this species is only recommended in areas with low rainfall and no access to irrigation.

6.8 Implications for Talish clover

There is currently only one available cultivar of Talish clover cv. Permatas, which has demonstrated high drought tolerance and persistence at several low rainfall sites across southern Australia (Hall 2013; Real *et al.* 2011). Successful establishment was achieved when this clover species was paired with winter active cocksfoot at 3 kg/ha and 5 kg/ha, and when paired with summer active cocksfoot at 3 kg/ha. The dry weights recorded for this species were low in comparison to that of red clover. The observed slow growth of this species during seedling development was in accordance with the results of other studies

(Dodd and Orr 1995; Hall 2013). Therefore, use of this species is only recommended in areas with low rainfall and no access to irrigation.

7 Conclusions and Further Research

The improved drought tolerance exhibited by Caucasian clover and Talish clover, make these species a suitable alternative for white clover in areas with annual rainfall below the threshold required for cultivation of this species (Nichols *et al.* 2012). This study confirmed the results of previous studies, highlighting the slow development and low productivity of Caucasian clover and Talish clover during early seedling growth (Dodd and Orr 1995; Black *et al.* 2006a). The results showed a lesser potential for light interception, and lower rate of biomass production by Caucasian clover and Talish clover, when compared to red clover and the two cocksfoot species. In spite of these differences, successful establishment of all three clover species was achieved when paired with cocksfoot at grass seeding rates within the breeders recommended range and under significant irrigation.

There were several factors that could have affected these results. At the field site there was a significant weed seed bank, and despite the application of herbicides prior to sowing, a significant population of wild radish (*Raphanus raphanistrum*) became established. The use of further herbicide applications was not possible after sowing due to the variety of pasture species included in the sward. Performance of grass and clover plants may have been negatively impacted during this period due to the limited availability of light. In the sixth week after sowing the site was mown down to 10 cm to allow light to reach the pasture seedlings. Some variation in the application of the irrigation was recorded at the site. There was a 25% difference in the amount of water applied to plots in one of the four blocks, which resulted in a lower plant density. Some of this variability was accounted for by the blocking, as only the plots in one block were significantly affected.

The results of this experiment highlight the need for further studies. A cost benefit analysis could be applied to compare the economic benefits of a lower grass seed usage, with the cost to biomass production. The validity of these results should be re-examined after grazing has been allowed on the site. There is limited data available that focuses on Talish clover. Further research could focus on its compatibility with other drought tolerant grass species such as phalaris, and its adaption to other environmental stresses such as salinity, which is also a problem in the Tasmanian midlands.

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9 Appendices

Appendix 1. The rate and duration of irrigation application for the duration of the experiment.

Date	Rate (psi)	Duration (minutes)
10/10/2017	50	50
17/10/2017	50	40
20/10/2017	50	40
23/10/2017	50	40
27/10/2017	50	50
1/11/2017	50	30
13/11/2017	50	15
20/11/2017	50	40
23/11/2017	30	25
23/11/2017	50	25
28/12/2017	50	30
04/01/2018	50	40
23/01/2018	50	60
02/02/2018	50	15
05/02/2018	50	20
20/02/2018	50	20
22/02/208	50	15

Appendix 2. Average weekly temperature (°C) for glasshouse over the course of the experiment ranged between 20 - 23°C.

Weeks after planting	Average weekly temperature (°C)
0	23
1	22
2	23
3	22
4	22
5	21
6	20
7	21
8	22
9	21
10	20
Average	22

Appendix 3. Seedlings replaced in glasshouse experiment one week after planting.

Pot identification	Number of plants replaced
P2. Caucasian clover	4
P27. Caucasian clover	2
P36. Caucasian clover	2
P34. Caucasian clover	3
P32. Red clover	1
P35. Caucasian clover	2
P11. Summer active cocksfoot	1
P31. Caucasian clover	3
P15. Caucasian clover	4
P6. Caucasian clover	2